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PREFACE

To the authors who made this volume possible, we gratefully offer two tokens: our sincere appreciation for their efforts and a special commendation for the absence of last-minute, defaulted manuscripts.

During this year Dr. Sterling Hendricks completed his term as a member of the Editorial Board. We are deeply indebted to him for his many valuable contributions. His successor is Dr. H. W. Siegelman.

The compilation of the manuscripts into a book was the responsibility this year of our very effective assistant editor, Miss Lois Cox; our subject indexer, Mrs. Grace MacNeill; and our printer, the George Banta Co.

We call the attention of the reader to the new indexes, started last year —The Cumulative Title Index and The Cumulative Author Index. With this volume these indexes assume their regular function of including the ten most recent publications in the series.

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CONTENTS

PAGE

PREFATORY CHAPTER:

SOME ASPECTS OF AMINO ACID SYNTHESIS IN PLANTS AND RELATED SUBJECTS, *A. I. Virtanen* 1

CELL STRUCTURE AND FUNCTION:

STRUCTURE AND FUNCTION OF MITOCHONDRIA, *F. L. Crane* . . 13

STRUCTURE AND PHYSIOLOGY OF CELL WALLS, *G. Setterfield and S. T. Bayley* 35

MINERAL NUTRITION:

MINOR MINERAL NUTRIENTS, *D. J. D. Nicholas* 63

SOIL-PLANT RELATIONSHIPS IN ION UPTAKE, *M. Fried and R. E. Shapiro* 91

NITROGEN METABOLISM:

PROTEIN SYNTHESIS, *G. Webster* 113

PHOTOSYNTHESIS:

PHOTOSYNTHESIS UNDER NATURAL CONDITIONS, *J. F. Talling* . 133

PHOTOSYNTHESIS, *G. Hoch and B. Kok* 155

RESPIRATION:

PHOSPHORYLATION IN HIGHER PLANTS, *E. Marré* 195

GENERAL METABOLISM:

BIOSYNTHESIS AND FUNCTION OF CAROTENOIDS, *T. W. Goodwin* . 219

BIOCHEMISTRY OF SENESCENCE, *J. E. Varner* 245

WATER RELATIONS:

PLANT WATER DEFICITS AND PHYSIOLOGICAL PROCESSES, *Y. Vaadia, F. C. Raney, and R. M. Hagan* 265

GROWTH AND DEVELOPMENT:

PHOTOPERIODISM AND THE FLOWERING PROCESS, *F. B. Salisbury* . 293

PHYSIOLOGY OF MITOSIS AND MEIOSIS, *J. H. Taylor* 327

INDOLE AUXINS, *C. H. Fawcett* 345

APPLIED ASPECTS OF THE GIBBERELLINS, *N. W. Stuart and H. M. Cathey* 369

KINETIN AND RELATED COMPOUNDS IN PLANT GROWTH, *C. O. Miller* 395

GEOTROPISM IN ROOTS AND SHOOTS, *H. Rufelt* 409

STRESS PHYSIOLOGY:

PHOTOCHEMICAL AIR POLLUTION DAMAGE TO PLANTS, *J. T. Middleton* 431

PATHOLOGICAL PHYSIOLOGY:

PHYSIOLOGY OF WILT DISEASE, *T. S. Sadasivan* 449

INDEXES:

AUTHOR INDEX 469

SUBJECT INDEX 488

CUMULATIVE INDEX OF CONTRIBUTING AUTHORS (VOLUMES 3 THROUGH 12) 496

CUMULATIVE INDEX OF CHAPTER TITLES (VOLUMES 3 THROUGH 12) 498

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Matti J. Virtanen

PREFATORY CHAPTER

SOME ASPECTS OF AMINO ACID SYNTHESIS IN PLANTS AND RELATED SUBJECTS

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The capacity of green plants and many microorganisms to synthesize their nitrogenous compounds using only inorganic nitrogen as a source of nitrogen is one of the fundamental phenomena of life. Because nitrogen occurs as an amino group in amino acids—the building stones of proteins—the problem of the formation of the amino group has attracted many scientists ever since the general structure of amino acids was elucidated. It is characteristic of the slight interest shown in plant biochemistry for many decades that theories on most of the important metabolic processes in plants, as for instance the biosynthesis of amino acids, were developed on the basis of experiments with animals. The theory presented by Knoop fifty years ago in which he postulated the reductive formation of amino acids from α-keto acids and ammonia was well founded chemically and strongly supported by animal experiments. This elegant theory could satisfactorily explain the biosynthesis of many amino acids as long as more detailed knowledge was not required of factors necessary for this synthesis. The rapid development of enzyme chemistry in the twentieth century led to the requirement that a proposed metabolic pathway in a biological system was hypothetic only as long as an enzyme necessary for it was not found in the system. Because no enzyme system which would cause reductive amination was known, even Knoop's theory was reduced to a hypothesis.

The first enzymatic synthesis of an amino acid from ammonia was of quite another type than the amination of a keto acid. At the end of the 1920's Quastel and Woolf (1, 2) discovered that resting cells of *E. coli* synthesized L-aspartic acid from fumarate and NH_4Cl. The reaction was reversible: L-aspartic acid$\rightleftharpoons$fumaric acid$+NH_3$. Some years later, from a dry preparation of *Pseudomonas fluorescens*, Tarnanen and I (3) obtained a cell-free extract containing the enzyme aspartase which causes this reaction. It was the first time an amino acid synthesis was performed with a cell-free enzyme solution.

Aspartase, which appeared to be strictly specific for L-aspartic acid, was found in many, but not all, microorganisms and also in some higher plants (3). The enzyme was not found in leguminous root nodules. It has never been found with certainty in animal tissues. Thus, it did not give rise to a general explanation of the amino acid synthesis from ammonia that occurs in all kinds of organisms. Aspartase was recently purified and characterized as a metalloenzyme in this laboratory by Ellfolk (4).

For many years aspartase was the only known amino acid synthesizing enzyme. In 1937–39 v. Euler & Adler (5), with collaborators, made the important discovery that glutamic acid dehydrogenase is specific for L-glutamic acid and that the reaction catalyzed by it is reversible: L-glutamic acid $\rightleftharpoons$ α-ketoglutaric acid + NH_3

The first step in reductive amination, i.e., the combining of ammonia with α-ketoglutaric acid, is not well understood even yet, but the first product was assumed to be α-iminoglutaric acid, which is then reduced by glutamic acid dehydrogenase to L-glutamic acid. This enzyme was found in all kinds of organisms: microorganisms, higher plants, and animal tissues. Later studies confirmed its decisive importance for the biosynthesis of L-glutamic acid. Knoop's idea of amino acid synthesis was thus realized in regard to L-glutamic acid, but at the same time it became evident that it was not a reaction that could explain the biosynthesis of the many different amino acids.

Two amino acids in which the amino group is formed through incorporation of an inorganic nitrogen compound into the carbon skeleton were thus established with certainty by the end of the 1930's. I have used the term "primary" for amino acids of this type; hence "secondary" amino acids refer to either those in which the precursor already contains the amino (or imino) group, or those to which the amino group is transferred from another compound.

The synthesis of other amino acids by an analogous mechanism was not known with certainty until quite recently, even though some evidence of the "primary" nature of alanine was presented earlier. In a scheme showing the synthesis of amino acids, ten years ago I used an arrow marked with a solid line for the biosynthesis of glutamic and aspartic acids from ammonia and an arrow marked with a broken line for alanine (6). The broken line however, can now be drawn as a solid one since Goldman (7) recently isolated and purified from a cell-free extract of *Mycobacterium tuberculosis* an alanine dehydrogenase which catalyses the reaction: L-alanine + DPN^+ $\rightleftharpoons$ pyruvate + NH_4^+ + DPNH.

It is probable that the mechanism of action of glutamic and alanine dehydrogenases is similar. An analogous mechanism has been proposed for some other amino acids, e.g., L-aspartic acid from oxalacetic acid and ammonia, but the enzyme preparations used were not purified and the reaction could not be sufficiently studied (8). Reductive amination of α-ketovaleric acid to L-norvaline, an amino acid which has never been found in higher organisms, has recently been carried out however, by crystalline glutamic acid dehydrogenase from liver (9). This reaction is possibly of no great biological significance, but from the biochemical point of view it is interesting, because it indicates that the γ-carboxyl group in α-ketoglutaric acid is not essential for the functioning of the enzyme.

Even if in some organisms alanine may be formed primarily from ammonia by alanine dehydrogenase, there is no proof that this amino acid

would generally be formed by this pathway. On the contrary, it is probable that alanine is of secondary origin in those organisms in which the amino acid synthesis has been studied in detail. In these cases it has not been possible to prove a primary formation of aspartic acid from ammonia. I refer only to some investigations with microorganisms and green plants here.

The experiments with ^{15}N, especially those which Burris, Wilson, and their associates have performed with nitrogen-fixing microorganisms using ammonium, nitrate, and molecular nitrogen as nitrogen sources, speak in favour of ammonia as the "key" compound and of L-glutamic acid as the only primary amino acid (10). The labelling of glutamic and aspartic acid has, however, in many experiments with molecular nitrogen been so nearly equal that aspartic acid could also be a primary amino acid, especially if it is formed both primarily and secondarily. Recent kinetic studies of Allison & Burris (11) using very short experimental times have, however, given conclusive evidence of the secondary origin of aspartic acid.

Investigations with low-nitrogen *Torulopsis utilis*, performed by Roine (12) in this laboratory, led to the conclusion that glutamic acid is the primary amino acid when the yeast is fed an ammonium salt. About 60 per cent of the soluble organic nitrogen formed during the first 10 min. was glutamine, 15 per cent glutamic acid, and 20 per cent alanine. The latter was assumed to be formed by transamination. Folkes (13) recently presented a kinetic analysis of his quantitative estimations of the incorporation of ^{15}N into the amino acids and proteins of *Torulopsis utilis*. The synthesis of glutamic acid and glutamine together accounted for at least 86 per cent of the probable synthesis of α-amino groups. Alanine was formed secondarily only as was probably also aspartic acid. The evidence for the last one was not as convincing as for alanine.

In green plants the synthesis of amino acids was studied by Rautanen (14); he used detached shoots of pea plants, cut just above the seed, and allowed them to take up various nitrogenous compounds through the cut ends. When fed ammonium nitrogen for 8 hr. the principal product was glutamine. Roughly 60 per cent of the total organic nitrogen formed was glutamine, 25 per cent glutamic acid, and 15 per cent asparagine. Rautanen also showed that in corresponding experiments nitrate was reduced to ammonia. At present there is no proof of different pathways for amino acid synthesis from nitrate and from ammonia (15).

According to our present knowledge it seems that the universal highway for incorporation of ammonia into the carbon skeleton to form amino acids is through the biosynthesis of L-glutamic acid. Thorough investigations are still needed to elucidate the biosynthesis of L-alanine and L-aspartic acid, the latter one especially in some of the organisms in which aspartase is to be found.

Another inorganic nitrogen compound, hydroxylamine, has been of great interest in connection with the biosynthesis of primary amino acids. Hydroxylamine is the last intermediate in nitrate reduction, and a specific

enzyme, hydroxylamine reductase, which reduces it to ammonia, has been demonstrated by many authors in recent years in different organisms(Egami, Nason, and Nicholas). As a very reactive substance, hydroxylamine forms oximes at a high rate with many carbonyl compounds $>CO+NH_2OH \rightarrow >C:NOH$. The reactivity of hydroxylamine with glyoxylic, pyruvic, oxaloacetic, and α-ketoglutaric acid is so great that hydroxylamine is fixed in the organisms *via* oxime formation if it is not immediately reduced to ammonia. Therefore, accumulation of free hydroxylamine in living cells is not probable. Hydroxylamine also forms hydroxamic acids —CONHOH. The enzymatic formation of γ-glutamyl- and β-aspartylhydroxamic acids both from glutamic and aspartic acids, as well as from glutamine and asparagine, has been demonstrated in many organisms (16, 17, 18). The enzymatic synthesis of benzohydroxamic acid has also been accomplished (19).

Other hydroxylamine derivatives seem not to be so uncommon among natural substances. Mustard oil glucosides, according to Ettlinger (20), can be considered as hydroxylamine compounds and a new type of glucoside which has recently been isolated in this laboratory from young rye, wheat, and maize plants (21, 22) can also be considered as substituted hydroxylamine compounds. Upon crushing the plants the aglucones of those glucosides, 2,4-dihydroxy-1,4-benzoxazine-3-one (21, 23) (from the rye glucoside) and its 7-methoxy derivative (22) (from the wheat and maize glucoside), are liberated enzymatically. From these, benzoxazolinone and 6-methoxybenzoxazolinone are formed by splitting of formic acid.

Many years ago Csáky and I (24) found a very rapid formation of "bound hydroxylamine" in a strongly aerated suspension of *Torulopsis utilis* in nitrate solution. The highest yield was usually obtained with nitrogen-deficient cells. Saris and I (25, 26) studied in detail this bound hydroxylamine fraction isolated by an ethanol extraction from *Torulopsis* cell mass after aeration in a sodium nitrite-containing solution. The hydroxylamine compounds extracted from roots of rye grown with nitrate and nitrite were also investigated. Amino acids were first separated quantitatively in an Amberlite IR-120 column. The oxime group was then reduced with sodium amalgam to an amino group, and the amino acids formed were identified and estimated by paper chromatography. In the root of rye the oxime of glyoxylic acid was most abundant, followed by the oximes of pyruvic, α-ketoglutaric, hydroxypyruvic, and oxalacetic acids. The corresponding keto acids could also be found in the roots in the same quantitative order as the oximes (27). In *Torulopsis* the oxime of pyruvic acid was quantitatively the highest, but oximes of α-ketoglutaric, oxalacetic, and glyoxylic acids were also formed.

Hydroxamic acids, which give a red colour with ferric chloride, were separated by paper chromatography. Acetylhydroxamic acid, which arises through a spontaneous reaction between hydroxylamine and acetyl compounds such as acetylphosphate and acetyl coenzyme A, could be identified, but no other hydroxamic acids (26). About 50 to 70 per cent of the hydroxylamine was found in oximes, the rest in acetylhydroxamic acid.

Through these investigations the "bound hydroxylamine" compounds formed in nitrate and nitrite reduction were chemically characterized for the first time. When ammonium nitrogen served as the source of nitrogen for *Torulopsis*, no bound hydroxylamine was formed (24).

If the oximes that were found were reduced in organisms, the corresponding amino acids could be formed from them. In that case the formation of many primary amino acids would be expected, not just glutamic acid as is the case when ammonia is the source of nitrogen. The tempting idea of the reduction of the α-oximino acids formed from α-keto acids and hydroxylamine to the corresponding α-amino acids could not, however, be substantiated. So far it has not been possible to obtain from *Torulopsis* or any other organism enzyme preparations active in the reduction of the above oxime compounds. The earlier mentioned possibility that amino acids also could be formed to some extent from the corresponding oximes with nitrate as the source of nitrogen, has not thus far been confirmed (28). The differences found between nitrogen fractions of *Torulopsis* grown with ammonium or nitrate nitrogen are only quantitative and depend mostly on the relatively higher rate of protein synthesis from nitrate than from ammonium nitrogen. The composition of the soluble nitrogen fraction was qualitatively similar in both cases.

Over thirty years ago Maurer (29) found, it is true, that alanine was formed when oximinopyruvic acid was added to an actively fermenting culture of brewer's yeast, but many unphysiological compounds are reduced by fermenting yeast. Oximinopyruvic acid was not reduced by a halotolerant coccus although it had some affinity for hydroxylamine reductase in experiments performed in Egami's laboratory (30). On the other hand, enzymatic reduction of aromatic *o*-nitroso and *o*-hydroxylaminobenzoic acids to *o*-aminobenzoic acid has been found in the same laboratory (31).

The rapid decrease of oximes in aerated *Torulopsis* suspensions makes it probable that some mechanism is formed in a short time by which the oximes are decomposed. A hydrolysis of oximes is quite possible, but is difficult to demonstrate because free hydroxylamine cannot be accumulated. Added oximinopyruvic or acetylhydroxamic acids did not disappear in *Torulopsis* cultures, and the organism was unable to use these compounds as a source of nitrogen in growth experiments. Obviously they cannot enter the cells.

Saris' and my results suggested that bound hydroxylamine arises as a by-product in nitrate reduction, the physiological function of these compounds being the "trapping" of hydroxylamine in case it is not immediately reduced to ammonia.

$$NO_3^- \rightarrow NO_2^- \rightarrow [?] \rightarrow NH_2OH \longrightarrow NH_4^+$$

$$\text{oximes, acetylhydroxamic acid} \quad \text{(not verified)} \quad \alpha\text{-amino acids}$$

On the basis of this assumption it can be understood that hydroxylamine

compounds are best formed in more or less extreme circumstances, for instance when the yeast is extremely deficient in nitrogen, whereby the activity of many enzymes, and possibly also of hydroxylamine reductase (not estimated), is lowered (32), or the concentration of nitrite is quite high (25, 27). Normally the hydroxylamine formed may be reduced when it is still attached to the reducing enzyme, thus leaving no possibility for the formation of free hydroxylamine.

Formation of hydroxylamine in biological nitrogen fixation has aroused special interest ever since Blom (33) found hydroxylamine in *Azotobacter* cultures when molecular or nitrate nitrogen served as the source of nitrogen. Before that the simplest and most probable pathway of biological nitrogen fixation was held to be a reduction of the nitrogen molecule to ammonia. It was expected that the formation of hydroxylamine could elucidate the chemical mechanism of nitrogen fixation on an experimental basis. Blom proposed the hypothesis that the nitrogen molecule was first hydrated and then reduced to hydroxylamine. Hydroxylamine however, can be formed from molecular nitrogen even in other ways. The first reaction stage could just as well be either reductive or oxidative. If it should be oxidative, nitrogen fixation could have a common intermediate with the reduction of nitrate even before the hydroxylamine stage (34). Considering all these reaction possibilities, even conclusive proof of hydroxylamine as an intermediate in nitrogen fixation would not solve the problem of the mechanism of nitrogen fixation, but it could essentially reduce the number of conceivable pathways.

In this laboratory the formation of bound hydroxylamine was observed in the 1930's in root nodules of leguminous plants as well as in *Azotobacter* cultures, and it was at first held possible that aspartic acid could be formed from hydroxylamine and oxalacetic acid via oximino acid (35, 36). Later, bound hydroxylamine was considered only as a by-product, hydroxylamine being reduced to ammonia. At the end of the 1940's, however, it was found that in anaerobic nitrogen fixation by *Clostridium* no traces of bound hydroxylamine could be detected (37, 38). There was thus no reason to assume that hydroxylamine was an intermediate in anaerobic nitrogen fixation, and a reductive pathway from the beginning to the end, without the formation of oxygen-containing intermediates was held probable in anaerobic nitrogen fixation.

A different mechanism for aerobic and anaerobic nitrogen fixation was not very likely, but as long as the formation of bound hydroxylamine in aerobic fixation was not explained, hydroxylamine could not be overlooked. Later investigations in this laboratory have led to results that justify our assumption of a similar mechanism for aerobic and anaerobic nitrogen fixation. It was shown that bound hydroxylamine is also formed from ammonia by *Azotobacter*. The rate of formation of these compounds in earlier experiments with *A. vinelandii* was slower from NH_4^+ than from N_2 and NO_3^- (37, 39), but in later experiments performed by Saris and me (40) with *A. chroococcum*, the rate was practically the same from all of these nitrogen sources.

Most important was the finding of an identical spot of bound hydroxylamine of a complicated nature on paper chromatograms, irrespective of which of the three nitrogen sources was used in the experiments (41).

Obviously ammonia was oxidized by *Azotobacter* to hydroxylamine. The reaction caused by hydroxylamine reductase of *Azotobacter* may be reversible, as shown by Klausmeier & Bard (42) with the corresponding reductase of *B. subtilis*. The formation of bound hydroxylamine is therefore no proof of the formation of hydroxylamine as an intermediate in nitrogen fixation. The entire reductive mechanism to ammonia is therefore possible in aerobic as well as in anaerobic nitrogen fixation. The investigations by the Wisconsin group have led to similar results (11). Since it has recently been possible to get cell-free extracts with an active nitrogen-fixing system from some N_2-fixing microorganisms (43, 44), new results which may give conclusive evidence of the mechanism of nitrogen fixation can be expected in the near future.

Secondary amino acids can be formed from primary amino acids by several very different reactions. The use of compounds labelled with ^{14}C has made it possible to elucidate the specific pathways of formation of many amino acids. At this time I will consider only the importance of the general reaction, transamination, for the synthesis of both secondary and primary amino acids and the presence of α-keto acids in plants, the indispensable components of transamination. Braunshtein & Kritsman (45) discovered this reaction in 1937 in animal tissues. Subsequently it was shown in this laboratory to function also in higher plants (46). The significance of transamination in living organisms has been much discussed. It was first believed to function only in systems where L-glutamic acid is the donor or α-ketoglutaric acid the acceptor of the α-amino group. Transamination was later demonstrated in many other systems of amino and keto acids. Not only dicarboxylic acids have turned out to be indispensable in the system, but an amino group even in other than the α-position has been found to be transferred as shown in this and some other laboratories in the system: γ-aminobutyric acid+α-ketoglutaric acid (47, 48). Also, Meister (49) observed the transamination of the α-amino nitrogen of glutamine and asparagine to a number of α-keto acids with the simultaneous splitting of the amide nitrogen to form ammonia.

Because transamination reactions are reversible, glutamic acid in the cell can at the same time be of primary and secondary origin. This can also occur with keto acids. They can be formed independently of transamination by different pathways (e.g., through condensation, carboxylation, decarboxylation, oxidation, etc.) or through transamination. For instance, oxalacetic and α-ketoglutaric acids are primary if they are formed via the Krebs' cycle, but secondary if they are formed by transamination from aspartic and glutamic acids, respectively. The equilibrium between these different components in such a complicated, dynamic system consisting of free amino and keto acids is regulated by many reactions. A most important one is without doubt transamination.

The central position of α-keto acids in the biosynthesis of amino acids

led us to develop methods in this laboratory for the determination of these acids. At the end of the 1930's we found the main α-keto acids—oxalacetic, α-ketoglutaric, and pyruvic acids—in higher plants (50, 51). Among the methods then used I may mention the transformation of keto acids to 2,4-dinitrophenylhydrazones, and the hydrogenolysis of these to the corresponding amino acids (52). The amino acids were then determined by different methods (51). After paper chromatography came into use in 1953, the identification of some new α-keto acids became possible as the 2,4-dinitrophenylhydrazones (53), but the hydrogenolysis of hydrazones to amino acids and their identification by paper chromatography (54) proved to be a more elegant method. Using the latter procedure, a large number of α-keto acids corresponding to known as well as new amino acids have been found in plants. During the last fifteen years so many new amino acids have been isolated, especially from higher plants, that it is not even possible to give the exact number of these compounds any more. These amino acids clearly illustrate how enormously versatile is the synthetic capacity of plants. Generally the new amino acids occur only in the free form in plants.

The close connection between amino and keto acids is revealed by the fact that in many plants in which new amino acids have been found, the corresponding α-keto acids can also be identified. Towers & Steward (54), who have done valuable work in this field, identified γ-methylene-α-ketoglutaric acid in the tulip where the presence of γ-methyleneglutamic acid was also shown. Done & Fowden (55) had recognized this amino acid earlier in the groundnut plant, and Fowden & Webb (56) identified the corresponding α-keto acid in this plant. The keto acid was also synthesized by transamination from γ-methyleneglutamic and α-ketoglutaric acids using an extract of groundnut seedlings as the enzyme preparation. In this laboratory α-aminopimelic acid, γ-hydroxy-α-aminopimelic acid, and its lactone were isolated from *Asplenium septentrionale* (57, 58, 59). The corresponding keto acids—α-ketopimelic acid, γ-hydroxy-α-ketopimelic acid, and its lactone—were found in the same plant (60). The formation of γ-hydroxy-α-ketoglutaric acid by transamination from α-ketoglutaric acid and γ-hydroxyglutamic acid was demonstrated with a homogenate of *Phlox decussata*, the plant in which this new amino acid was first found (61, 62). Numerous keto acids have been identified in different plants in this laboratory.

There is thus experimental evidence for the opinion that many amino acids are formed in plants by transamination. Naturally, the presence of the keto acids and the corresponding amino acids in the same plant is no proof of transamination reactions *in vivo*. Considering, however, that the transaminases catalyzing these reactions are also present in the plant, the idea concerning the importance of transamination in the synthesis of amino acids in plants is well founded.

The ability of higher plants to take up organic substances through their roots is a problem that has interested me since the beginning of the 1930's. Our experimental investigations have concentrated on the uptake of nitrogen-

containing organic substances. In growth experiments performed in sterile nutrient solution, pea plants and red clover were found to grow as well with L-aspartic acid as the only source of nitrogen as with nitrate nitrogen (63, 64, 65). Good growth was later obtained with L-glutamic acid and L-alanine, too. The D-form of DL-aspartic acid was taken up to some extent, but only the L-form was used in growth (66). With other amino acids, except L-arginine, there was no growth of pea plants beyond that of plants given no nitrogen. Although several of the amino acids (L-histidine, L-lysine, D- and L-valine) were taken up in relatively large amounts and the nitrogen content of the plants increased, nevertheless, the plants did not grow (on a dry matter basis).

To prove that aspartic acid was really taken up as such, and not deaminated on the root surface, the pH of the nutrient solution was followed during the experiment. No decrease in the pH, which would be expected if the plant took up only the amino group from aspartic acid, could be observed during the 6-week experiment (63, 67). The ratio of carbon to nitrogen, which is 3.43 in aspartic acid, did not change in the aspartic acid-containing nutrient solution. On the basis of these observations I concluded that the pea plant takes up the whole aspartic acid molecule and thus gets the carbon skeleton of the amino acid. Aspartic acid competed successfully with nitrate and ammonium nitrogen as the source of nitrogen for the pea plant (68). From a sterile nutrient solution which contained the same amounts (50 mg.) of aspartic acid-N, NO_3-N, and NH_4-N, the pea plant took up 40.0 mg. of amino-N, 40.8 mg. of NH_4-N, and 25.8 mg. of NO_3-N during a growth period of 28 days.

In corresponding experiments with barley and wheat it was unexpectedly found that they were not able to utilize L-aspartic and L-glutamic acid, whereas L-arginine and L-alanine were used to a considerable extent (64, 66). At present we have no explanation to offer for the different utilization of aminodicarboxylic acids by the leguminous and grass plants used in the experiments.

With 1-^{14}C-labelled DL-alanine, Miettinen (69) recently studied the uptake of this amino acid from a nutrient solution by pea plants. After 3 hr. the labelled alanine was found in the root and in the shoot. It was the only amino acid in which activity could be detected. The experiment with 1-^{14}C-L-alanine gave similar results. When 1-^{14}C-DL-glutamic acid was used, about two-thirds of the activity was found in glutamic acid and one-third in alanine. The uptake of amino acid molecules by plants thus had been definitely shown.

In the 1930's the idea that plants were able to take up amino acids and, further, in amounts sufficient to replace normal inorganic nitrogen nutrition was quite new. When the pea plant grows with aspartic acid nitrogen alone, the carbon skeleton obtained by the plant corresponds to about 20 per cent of the total carbon in the plant (respiration is not considered). The plant has thus become partly heterotrophic in regard to carbon.

Some amino acids and their decarboxylation products have been found to cause profound morphogenetic effects on plants. DL-alanine in nutrient solution made pea plants bushy and branching, and their root systems short and tuftlike (68, 70). Miettinen (69) later found that only D-alanine is toxic. Phenylethylamine, the decarboxylation product of phenylalanine, added to a nitrate-containing nutrient solution in amounts of about 0.05 per cent, caused strange changes in the pea plant (68, 70). Steinberg (71) has noted that isoleucine and leucine in quantities of 0.002 to 0.01 per cent caused changes in tobacco seedlings resembling those of the "frenching" disease. Only the L-forms of the amino acids were toxic. The most drastic morphological changes caused by an amino acid (glycine) were recently observed by Waris (72) in *Oenanthe aquatica*. In neomorphic plants of this species Miettinen & Waris (73) found ten times more soluble nitrogen than in normal plants.

Foreign organic substances that have passed into the plant in very small amounts from the substrate may perhaps have a morphogenetic effect even under natural conditions. According to our experiments, plants take up these substances (amides, alkaloids, etc.) much more easily from a nutrient solution than from soil. The whole problem urgently needs further elucidation.

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STRUCTURE AND FUNCTION OF MITOCHONDRIA[1,2]

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Some sort of exponential growth curve would best describe the increase of papers on the various aspects of mitochondrial function in the past few years. Fortunately for this author the number of reviews on the subject shows a proportionate increase. Since several very complete reviews on mitochondria have appeared recently I have not attempted to catalog all events, but have tried to concentrate on certain developments that have increased our understanding of the structural and functional relationships in mitochondria. In other areas I have concentrated primarily on studies of plant mitochondria. Of course most of the work reported in the literature involves animal mitochondria. Detailed investigation of plant mitochondria is still rather meager, but shows signs of increasing. It may be that there is some feeling that work on plant mitochondria is rather unnecessary since studies on animal mitochondria have been so extensive; after all, mitochondria are mitochondria so why struggle with plant material to extend to the plant kingdom what is already accepted truth based on animal studies.

Of course the answer to these objections lies in the fact that plants carry on different functions than animals, some of which may depend on unique mitochondrial systems. Also, we must consider the possibility that the lipoprotein structures of plant cells that can function happily (we suppose) either in ice banks or in tropical deserts display characteristics possibly not found in the pampered lipoprotein structures of the constant-environment warm-blooded animal system where at least 90 per cent of mitochondrial studies, and for that matter studies of any lipoprotein systems, have been concentrated. In any event, differences between plant and animal mitochondria are beginning to show up (e.g., the b_7 cytochrome) and more can be expected as the detailed study intensifies.

The following reviews have appeared recently and provide overall coverage of the various aspects of mitochondrial function to about 1958.

Schneider (1) presented an extensive review of mitochondrial metabolism with emphasis on the various enzymic functions and Green (2) wrote a thorough discussion of the electron transport system. Green & Lester (3) discussed the role of lipids in electron transport. The significance of structural and functional relationships has also been discussed in several articles (4, 5, 6, 7). Slater (8) has reviewed studies on heart muscle mitochondria. Hackett (9) has discussed respiratory mechanisms in higher plants. Oxidative phosphorylation has been discussed by Boyer (10) with respect to oxygen isotope

[1] The survey of literature pertaining to this review was concluded in October, 1960.

[2] The following abbreviations are used: DNP (dinitrophenol); DPN (diphosphopyridine nucleotide); DPNH (diphosphopyridine nucleotide, reduced form); TPNH (triphosphopyridine nucleotide, reduced form).

exchange and by Lehninger (11) in relation to swelling and contraction phenomena as well as in fragmented systems (12). The status of cytochromes in general has been reviewed by Slater (13) and in higher plants by Smith & Chance (14).

STRUCTURE

Mitochondria have much in common structurally with other cytological bodies in that they are framed by lipoprotein layer systems otherwise described as membranes. Most studies defining the membranous nature of both the outer envelope and inner framework of mitochondria have been made with the electron microscope as originally described by Palade (15) and Sjostrand (16). The structural studies have been discussed by Andersson-Cedergren (17). Observations on structure have been supplemented with electron microscope observation of experimentally induced changes in membrane structure, e.g., by the deoxycholate treatment of Watson & Siekevitz (18), and with ethanol plus phosphate by Ziegler *et al.* (19) [see also Pauly, Packer & Schwan on electrical properties (20)].

A strange and yet unexplained feature of the lipoprotein membrane systems of cells lies in the characteristic double membrane phenomenon observed following fixation with osmic acid and embedding in plastics. Mitochondria universally show this double membrane structure both in the external membrane and in the cristae or internal membranes as observed by Sjostrand (16). It might be well to ask again what is the significance of the double membrane structure and is it a fixation phenomenon or does it in fact represent a two layer system? Revel, Ito & Fawcett (21) have demonstrated biphasic membranous systems using phospholipid embedded in plastic with staining by permanganate or osmate, thereby indicating that protein is not required in such structure.

The duality has not been demonstrated in other than fixed systems (22). In fact, membranes from which the embedding material has been removed do not show double structure. Stoedsenius (23) has presented an interpretation of osmium fixation in myelin figures that may be pertinent to dual membrane phenomena.

On the other hand, experimental demonstration that a real duality exists in the mitochondrial membranes comes from the work of Ziegler *et al.* (19) in which the separation of the dual membrane structure (or the destruction of that structure) by simple chemical or physical treatments can be shown, and separation of the membranous vesicles that possess only single membrane structure is also shown. Whether this change is brought about by twisting of the double structure to form nodes, accompanied by swelling of the internodal sections to form vesicles, or whether the treatment destroys one layer of the double system selectively is not known [cf. Hodge (24)].

More sensitive and selective methods are needed for study of membrane structure as are studies of the basis for selective staining in electron microscopy. One step in evolving a selective assay of lipoprotein membranes is seen in the development of an antibody preparation to detect the presence of

minor protein units and their location in the structure, and to determine the fate of these structural proteins as the membrane structure is altered (25).

A unique feature of mitochondrial structure, which is the basis for identification of mitochondria in tissue sections, is the internal membrane or cristae (15, 16). Whether the cristae have the same structure and function as the external mitochondrial membrane is unknown. One interpretation may be that the formation of cristae is a device to extend the total lipoprotein membrane volume within the mitochondrial limits. It has been noted that the density of cristae is proportional to the concentration of cytochromes and other electron transport functions. Whereas the mitochondria of liver, which perform many ancillary functions other than the basic processes of Krebs cycle oxidations, show much intercristeal space, heart mitochondria primarily involved in ATP formation are densely packed with cristae with little space between.

Furthermore, it has been noted that oxidative phosphorylation follows the cristae during separation of mitochondrial fragments and that the lighter vesiculate material does not phosphorylate (19). It might be well to consider whether the external membrane is functionally different even though strongly resembling the cristae in composition. A parallel situation may exist in chloroplasts with a clearly differentiated granum and external membrane system (26). Zebrun & Mollenhauer (27), using potassium permanganate fixation of rat testes, observed rather striking differences between cristae and the external membrane with respect to depth of staining. They also observed a different staining behavior in mitochondria of different cell types and have noted in certain cells dense black bodies that are more analogous to the carotenoid-containing granules of beef heart or the lysosomes of liver (28) than to mitochondria.

There is also evidence that not all cristae are built the same. Willmer (29) has suggested that tubular processes or microvilli in mitochondria, characteristic of certain steroid-producing cells and specific lower animals as well as certain plants [cf. Hackett (9)], are more primitive than the usual lamellar system that is so well organized in higher animals, especially in muscle tissue. Von Wettstein (26) also shows differences between the lamellar structure in proplastids and leaf mitochondria and a minimum of cristae in the leaf mitochondria. Here again, there seems to be a correlation between mitochondrial structure and predominant function. In other words, mitochondria built to supply massive amounts of ATP for muscle contraction differ structurally from those engaged in keeping a leaf cell alive. The electron micrographs by Hodge *et al.* (30) of wheat root mitochondria also show a minimum of cristae.

The Swelling Phenomena

It has been recognized for some time that the structure of mitochondria is altered when they are exposed to hypotonic solutions and the swelling so induced has been shown by Tedeschi & Harris (31), also (32, 33), to be related to membrane permeability. There is an increase in total volume as well

as an increase in the volume of intercristeal space. This swelling is related functionally to certain of the "opening phenomena" described. These phenomena involve the appearance of certain enzyme activities hidden in the intact mitochondrion (34, 35). The primary enzyme function that appears coincident with swelling of beef heart mitochondria is the ability of the mitochondrion to oxidize external DPNH and citrate (2). De Duve and coworkers (34) have described other "openings" in liver mitochondria. An opening effect of this type might best be related to a permeability change that permits the substrate to get to an active site. However, the alternative hypothesis that these enzyme functions appear because of an uncoiling or unfolding of the surface lipoprotein structure has not been eliminated. Pauly & Packer (20, 36) have examined the electrical capacity (by measuring the dielectric constant of mitochondrial suspensions) of mitochondrial membranes and find that it does not change over a fourfold range of mitochondrial size induced by osmotic swelling. Conductivity studies indicate that swollen mitochondria tend to equilibrate with the external medium, whereas shrunken mitochondria tend to maintain a constant ionic composition regardless of changes in external ionic strength. This indicates a relatively large, osmotically active space in the swollen condition, but a predominant binding of charge by macromolecules in the shrunken condition. These authors suggest constant replacement of the inner membrane by material from the matrix during swelling.

More extensive "opening phenomena" are encountered as mitochondria are treated with detergents. The classic opening effect of deoxycholate on the cytochrome-*c* reductase of the electron transport system from beef heart mitochondria is an example of an effect that is accompanied by extraction of material from the mitochondria (lipoprotein in this case) and can best be interpreted in terms of exposure of an active site, something like scraping butter off toast (37).

With our meager knowledge of lipoprotein structure and the effects of external materials on that structure, all opening effects certainly cannot be assumed to be related to permeability changes in a bag holding a soup of enzymes. Watson & Siekevitz (18), in their electron microscope study of progressive degradation of mitochondria under the influence of detergents, indicate that inner contents are lost after extensive expansion of the membrane. It has been our experience that there is a successive extraction of enzyme units with an increasing concentration of detergent (38).

Swelling and Contraction Associated with Phosphorylation

Oxidative phosphorylation has been regarded until recently as a rather labile process closely allied to mitochondrial "integrity." Harmon & Fiegelson (39) were able to show that loss of integrity paralleled swelling of mitochondria. Subsequently it was found that ATP was lost during swelling and the addition of ATP and magnesium ions would reverse degradative swelling of mitochondria (40, 41). Since these initial studies, extensive work in a

number of laboratories has introduced numerous swelling agents into the field and in some instances the results seem to imply that several mechanisms for swelling exist. A detailed discussion of all of these effects and the conditions required to induce and inhibit swelling cannot be attempted here. It will be more useful for the purposes of the review to discuss certain recent papers that in themselves present a fairly good outline of the present status of the problem.

Lehninger (42) has outlined the various factors that induce swelling in rat liver mitochondria. These include thyroxine, phosphate, arsenate, calcium ion, exposure to hypotonic media, incubation at 37°C. (spontaneous swelling), *p*-chloromercuribenzoate, phlorizin, *U* factor [which apparently is an unsaturated fatty acid released by lipase action from mitochondria since oleate shows similar effects (cf. mitochondrial integrity below)], glutathione, carbon tetrachloride, digitonin, and zinc ion. He has been able to show that the swelling induced by most of these agents can be reversed by ATP, magnesium ion, serum albumin, and ethylenediaminetetraacetic acid in various combinations. Only swelling induced by glutathione, carbon tetrachloride, and digitonin could not be reversed by some combination of the above reagents. It is proposed that the reversal of swelling by ATP, together with the additional factors mentioned, involves a single fundamental mechanism.

Hunter *et al.* (43) have examined the swelling produced by an oxidizable substrate and oxygen, especially with reference to the agents that will inhibit this swelling. Their results all point to the general conclusion that an oxidation-reduction process at one or more points in the electron transport chain is associated with the swelling process whether or not phosphate is present. These authors, as well as Lehninger, point out that swelling induced by glutathione, cysteine, and ascorbate is manifestly different than that associated with other substrates, although oxygen is necessary in all instances. Their discussion of metal chelation and the oxidation of sulfhydryl groups in the mitochondrial membrane seems highly pertinent to possible mechanisms for the swelling reaction. Some reports, however, indicate that reducing agents prevent swelling (44).

Chappell & Greville (45) showed that swelling induced by substrate oxidation in the presence of phosphate does not directly involve coupled phosphorylation. This conclusion is primarily based on the relative inhibitory effects of oligomycin, amytal, and antimycin-*A* on swelling induced by electron transport from substrates to ferricyanide or to oxygen.

Packer (46) studied the swelling induced by substrate oxidation in the presence of phosphate and showed that ADP causes shrinkage of the swollen mitochondria, and that the shrinkage is followed by expansion as the ADP is converted to ATP. Lehninger (42) reported ADP effective in reversing only the swelling induced by hypotonic media, or by *U* factor or oleate and suggested that these conditions are auspicious for adenylate kinase activity to produce ATP. Packer's studies develop a further point of interest in that

reduction of cytochrome-*b* is shown to precede swelling, whereas oxidation accompanies shrinkage. Overall electron transport increases when ADP is added as shrinkage begins. Thus the primary act leading to swelling may involve a reduced rather than an oxidized state of the key compound. More recently, Packer & Tappel (47) have extended to mitochondrial fragments the swelling studies associated with phosphorylation. Lehninger & Gotterer (48) have reported a protein fraction required for mitochondrial contraction.

These extensive studies on mitochondrial swelling and contraction have been concentrated on animal tissue. Studies with plant mitochondria would provide useful comparative data and could help to emphasize aspects of general significance. It would also be intriguing to know if plants possess a contractile protein in the mitochondrial membrane or if permeability changes alone account for swelling phenomena.

Knowledge of swelling changes in plant mitochondria might also apply to physiological studies. The type of external conditions that effect ageing changes in potato slices as reported by Hackett *et al.* (49) are similar to the conditions that affect mitochondrial swelling. Thus they report that the ageing changes are inhibited by cyanide, dinitrophenol, and anaerobiosis. Mitochondria from the aged tissue show remarkable increases in the activity of certain enzymes that are suggestive of permeability changes. Key *et al.* (50) show swelling of mitochondria and increased enzyme activity after 2,4-dichlorophenoxyacetic acid treatment of soybean seedlings.

ATPase activity is also often associated with swelling of mitochondria. Maley & Johnson (51), however, have shown that agents that cause swelling do not always increase ATPase activity. There remains a group of compounds, however, that cause both swelling and an increase in ATPase activity. The possibility that the effects of these compounds relate to a site common to both processes cannot be ignored. Calcium, thyroxin, fatty acids, and cysteine seem to be the compounds of primary interest in this regard. Since DNP stimulates an ATPase reaction but inhibits swelling (52), it cannot fit into the above group. This is consistent with the indication by Lipsett & Corwin (53) that the effects are independent. Forti (54) has shown that calcium ion stimulates ATPase activity as well as swelling in pea mitochondria.

The relation of ATPase and phosphate-ATP exchange reactions in mitochondria to the process of oxidative phosphorylation has been reviewed (55, 56), and further discussion will be left to the review of phosphorylation reactions.

Formation of Mitochondria

The studies of Chevremont & Frederic (57), showing the dynamic state of mitochondria in fibroblasts, open up new considerations in any interpretation of mitochondrial structure. The rigid structure observed in fixed sections in electron microscope work may actually be misleading since it implies a constancy of composition that is impossible. It is obvious that to undergo increase and decrease, and breaking and fusion, mitochondria must con-

stantly change in composition and in the state or phase of the structural material. These considerations must be related to the studies of swelling and contraction discussed above, and in particular to the work of Pauly & Packer (20, 36) in their suggestion of constant replacement of inner membrane by material from the matrix during swelling. The effects of ATP and ADP on mitochondrial volume, discussed in relation to swelling phenomena, are also pertinent to structural change and how it may occur.

Another aspect concerning the formation of mitochondria involves the increasing number of reports of the sudden appearance of mitochondria during development. It would seem that mitochondria are present in largest numbers during differentiation. Gustafson (58) has reported studies on sea urchin eggs and Lund *et al.* (59), Ts'o & Sato (60), and Hanson *et al.* (61) have followed mitochondria during the development of seedlings. Brand & Mahler (62) have continued studies on mitochondrial enzymes in chick embryos. Lund *et al.* (59) also show a progressive increase in the number of cristae per mitochondrion during maturation of root tip cells of corn (*Zea mays*). Meristematic cells show mitochondria with hardly any cristae, but the cells are rich in cytoplasmic granules.

The work of Simpson and co-workers (63, 64), and others (65), has made it clear that mitochondria can synthesize at least part of their protein complement. Iron can be incorporated into cytochromes (66). Kennedy (67) has shown phospholipid synthesis goes well in mitochondria. Kahn & Hanson (68) report both phospholipid synthesis and destruction in corn mitochondria.

Weinbach & Garbus (69), in a study at the other end of the developmental scale, have found a decline in phosphorylative efficiency in mitochondria from aged rats that is partially associated with increased instability of mitochondria. In studies of wheat root mitochondria, Lindblad (70) has suggested that the instability of mitochondria in wheat roots grown at a low calcium concentration may account for the increased recovery of mitochondria from roots when calcium is present. This had been observed previously by Florell (71). It is intriguing to consider whether the effects of calcium on wheat root mitochondria have some mechanistic connection with the rather specific uptake of calcium by potato mitochondria reported by Shean & Levitt (72). Akazawa & Beevers (73) have followed the increase and then the breakdown of mitochondria in germinating castor bean endosperm. Thus the evidence begins to show that mitochondria can build themselves up and tear themselves down with abandon, and it leaves us with a rather dynamic picture of mitochondrial structure.

Binding of Pyridine Nucleotides

Intact mitochondria of animal tissue contain considerable amounts of bound pyridine nucleotide. The bound pyridine nucleotide can be released from or destroyed in mitochondria by exposure to aerobic conditions or to phosphate buffer (74, 75, 76).

A supply of ATP or an anaerobic condition is necessary to maintain the nucleotide in a bound form. Also, the presence of ethylenediaminetetraacetic

acid in the incubation medium prevents loss of bound phosphopyridine nucleotide.

In a study of conditions necessary for rebinding DPN, Hunter *et al.* (75) find that the oxidized form rebinds in the mitochondrial structure better than does DPNH, but it is not clear whether the same binding sites are involved as in the original attachment of DPNH. They point out that a more precise localization of binding sites will be necessary to understand the mechanism of the process.

FUNCTION

Citric Acid Cycle Oxidations

Evidence for operation of the tricarboxylic acid cycle in plant mitochondria has been reviewed several times (9). This evidence continues to be extended to tissues in which difficulties had been encountered and to new material (77, 78, 79). Results so far are strongly in favor of the usual tricarboxylic acid oxidation pattern in plant mitochondria.

Oxidative phosphorylation steadily improves in plant preparations until now in many cases it has reached the levels found in animal systems that closely approximate theoretical values (9, 49, 61, 73, 80, 81). In most cases the system acts like the animal system, but extensive study of oxidative phosphorylation in plant particles (as conducted with liver and heart mitochondria) has not been done. In some instances the inhibitors of the system encountered in animal studies are not effective (80).

Inhibitory effects of CO_2 on castor bean mitochondria have been reported (82, 83). Hackett *et al.* (84) studied the comparative effects of inhibitors on phosphorylation and electron transport and have shown (49) that cyanide uncouples phosphorylation in potato mitochondria.

Low sensitivity to DNP uncoupling has been reported and needs further examination (85). Usually DNP has acted as an efficient uncoupler with plant mitochondria (9, 86) and Gaur & Beevers (87) present evidence that many substituted phenols function as uncouplers in carrots. It should also be of interest to know if the animal hormone thyroxin uncouples phosphorylation in all plant mitochondria as it does in yeast (88).

The action of ribonuclease (89) in uncoupling phosphorylation in both plant and animal mitochondria raises a question of polynucleotide function in phosphorylation, as suggested by Pinchot (90, 91), or of a possible uncoupling action of the products after digestion.

Electron Transport

The electron transport system of plant mitochondria appears to be similar to that of various animal systems. Spectrophotometric evidence indicates that flavoproteins and cytochromes of the *b*, *c*, and *a* type participate in the oxidation of DPNH and succinate. In most cases the system is susceptible to the same inhibitors as animal systems. Antimycin and heptyl quinoline N-

oxide, azide, cyanide, and carbon monoxide usually cause inhibition at expected levels. Certain plant systems, however, possess a cyanide-insensitive respiration that is indicative of other than the usual cytochrome-a-a_3 terminal oxidase system. Perhaps the best interpretation so far advanced to explain this situation is based on the existence of a special auto-oxidizable b cytochrome that can link the nonphosphorylating electron transport from substrate to oxygen [Bendall & Hill (92)]. This may not be the final answer, however, since Chance & Hackett (93) presented evidence that no unusual pathway branches off in skunk cabbage. It is this author's conviction that the components of plant mitochondria are not sufficiently inventoried to preclude surprises in the nature of unknown electron carrier molecules. Even in animal mitochondria the studies of Thorn & Jackson (94) indicate separate unknown sites for the action of antimycin-a and BAL (2,3-dimercaptopropanol).

Much remains to be accomplished in isolating the members of the electron transport chain from plant mitochondria. Although there is general agreement that plant systems possess cytochromes of the a, b, and c series, it is not clear how closely related they are to the corresponding cytochromes of mammalian tissues.

Lundegardh (95) presented spectra from wheat roots indicating, by different reduction rates for cytochromes with absorption bands at 550, 553, and 563, the presence of cytochromes c, c_1, and b respectively.

On the other hand, Chance & Hackett (93), in a study of *Symplocarpus* mitochondria, found a cytochrome-c band at 551mμ, a b cytochrome with an α band at 558 mμ (which they refer to as b_7 although they point out that it differs from b_7 as previously described by Bendall & Hill). After dithionite treatment, they find indications of an α band at 567mμ that cannot be equated with any known cytochrome-b.

Bendall & Hill (92) indicated in their study of *Arum* that absorption bands of cytochrome-b_7 (557.5) and b (563) were present in that tissue. Evidence for c, by low temperature treatment in *Arum* mitochondria, has been reported (96). Sisler & Evans reported cytochromes of the b and c type in tobacco root preparations (97) and Baker & Lieberman (98) found evidence for four cytochromes in sweet potato mitochondria that would seem very close in absorption maxima [605 (a), 563 (b), 558 (b_7), and 551 (c)] to mammalian cytochromes except for a slight shift of the cytochrome-c and the 558 band, indicative of the presence of b_7 rather than c_1. In potato mitochondria Hackett *et al.* (49) found difference spectra similar to those mentioned above with a b_7 type band rather than c_1.

Of course the unusual respiratory characteristics of *Symplocarpus* and *Arum* spadices are appropriate to unusual cytochromes especially if the presence of b_7 type compounds can explain cyanide-insensitive respiration.

In comparing mitochondria from fresh cut potato slices with those from 24-hour-old slices, Hackett *et al.* (49) indicated that development of cyanide-

resistant respiration in this tissue during ageing depends on a metabolically induced modification of the respiratory chain. They show development of cyanide insensitive succinoxidase in mitochondria during ageing, as well as a large increase in cytochromes-*c* and -b_7 relative to the cytochromes-*a* and -a_3.

A system analogous to the cyanide-insensitive respiration in plants is found in the flavin-linked oxidative enzymes of *Lactobacillus* which have been studied by Strittmatter (99). A new terminal oxidase, cytochrome-*o*, has been reported by Castor & Chance (100) in certain bacteria.

It would seem that the apparent lack of the usual cytochromes in certain

TABLE I

EXTRACTION OF CYTOCHROMES

Cytochrome	Source	Reagents Used*	Reference
c	pig heart	acetone and salt	(187)
c_1	beef heart	detergents	(188)
c_4	*Azotobacter*	butanol	(189)
c_5	*Azotobacter*	butanol	(189)
b	beef heart	detergents	(190)
b	beef heart	detergents	(191)
b_2	yeast	autolysis	(192, 193)
b_5	rabbit liver	lipase digestion	(195)
b_8	leaves	soluble	(194)
a	beef heart	detergents and thioglycollate	(106)
f	parsley	alkaline ethanol	(196)

* Reagents used to extract cytochromes in procedures that lead to a highly purified enzyme. Other methods used for isolation of cytochrome-*c* in plants have been reviewed by Hill & Hartree (202).

plant tissues would call for a further development of this subject. If new cytochrome combinations exist in plant tissues, careful and precise spectrophotometric examination of new plant tissues, as well as isolation of the cytochromes, will be necessary to establish their identity. The isolation of various cytochromes from animal tissue and microbes (cf. Table I) indicates that the isolation of corresponding compounds from plant tissue can be accomplished, especially if the operation is done on a sufficiently large scale.

There seems to be general agreement that the cytochrome a–a_3 group with bands at 445 and 603 mμ exists in plants although, as Chance & Hackett (93) point out, cytochrome oxidase activity seems out of proportion to the amount of cytochrome-a_3 present.

In studies of cytochrome oxidase in plants it would be worthwhile to consider the evidence that copper is associated with the purified oxidase in

animal mitochondria (101); it has been shown by Wainio *et al.* (102) to be present in proportion to the oxidase activity during purification. Evidence for oxidation-reduction of the copper has been presented (103, 104). It may well be that the copper unit in the oxidase system is present in plants in larger amounts, relative to cytochrome-*a* or -a_3, than in animal mitochondria. In the isolation attempts so far reported it looks as if the cytochromes-*a* and -a_3 in plants [perhaps better called oxidase unit until a_3 is shown as a discrete compound (105), cf. Ambe & Venkataraman (106)] are not easily extracted at least by the procedures that are so successful with animal mitochondria (107, 108).

Giuditta & Singer (109) have studied the relative efficiency of various electron acceptors with animal preparations containing succinic dehydrogenase activity and find considerable variation in the rate of reduction with different acceptors. Phenazine methosulfate was the only one to react at a rate comparable with that of the overall oxidase system. These results are pertinent in any attempt to assess overall *in vivo* dehydrogenase activity by use of *in vitro* acceptor systems.

Ziegler, Green & Doeg (110) have described a lipoflavoprotein diaphorase from heart mitochondria that reacts more efficiently with ferricyanide than other diaphorases or cytochrome-*c* reductase. There is also an accumulation of evidence indicating that certain diaphorase activity may more accurately represent lipoyldehydrogenase (111, 112, 113). The evidence that fatty acyldehydrogenases can be released from heart mitochondria by sonic treatment (110) would indicate that the electron transferring flavoprotein and its accompanying diaphorase (114) may also be expected in mitochondria. Thus activity designated as diaphorase activity may represent the presence of at least three different enzymes in mitochondria (cf. Figure 1). In addition, Chance & Hackett (93) report an unusual amytal-insensitive system.

Cooperstein (115) has described direct reduction of cytochrome oxidase and cytochrome-*b* by a DPNH—cytochrome-*c*—reductase preparation. His preparation of DPNH—cytochrome-*c*—reductase also contains the mysterious 408 compound (oxidized max. 408 mμ, reduced 430 mμ) which has turned up in many studies of DPNH—cytochrome-*c*—reductase systems (116) (reported as cytochrome-*c*) (117) but has usually been dismissed as a degradation product. In his studies Cooperstein also does not take into account the evidence that a lipid-cytochrome-*c* complex is the active form of cytochrome-*c* (118, 119).

Sisler & Evans (120) have reported the purification of a diaphorase from the soluble fraction of tobacco root homogenates that is equally active with either DPNH or TPNH as the electron donor. In this respect it resembles the electron transport flavoprotein diaphorase from pig liver mitochondria (114). It is noteworthy that they find other diaphorase activity remains in the insoluble (mitochondrial?) fraction (cf. 121), but they do not indicate whether the particle-bound diaphorase is active with both nucleotides.

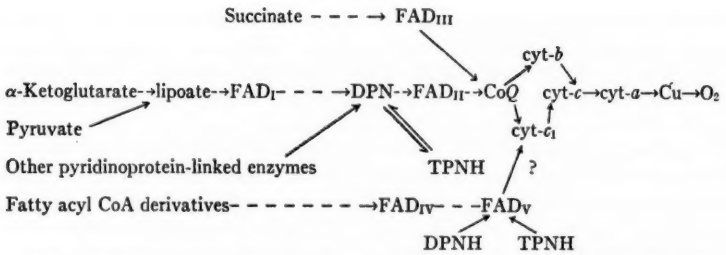

FIG. 1. The flavoprotein situation in mammalian mitochondria, showing the pathways of probable electron or hydrogen flow from substrates to oxygen. This is a summation of pathways reported from diverse mitochondria (see text). The following symbols are used: FAD_I (diaphorase, also lipoyldehydrogenase); FAD_{II} (DPNH-cytochrome-*c* reductase); FAD_{III} (succinic dehydrogenase); FAD_{IV} (acyldehydrogenases); FAD_V (electron transferring flavoprotein); FAD (flavoprotein); Co*Q* (coenzyme *Q*); (cytochrome with individual cytochromes designated by letter); Cu (copper); and O_2 (oxygen). Other flavoproteins such as α-glycerophosphate dehydrogenase have been omitted.

LIPID CYTOCHROME-*c* AND THE INTEGRITY OF ELECTRON TRANSPORT SYSTEMS

When the electron transport capacity for the oxidation of succinate or DPNH is examined in mitochondria and mitochondrial fragments from various sources, it is immediately apparent that some preparations require addition of cytochrome-*c* for maximum activity whereas other preparations show no cytochrome-*c* requirement. The electron transport particle of beef heart mitochondria (122) and that from *Azotobacter vinelandii* (123) contain tightly bound cytochrome-*c*, whereas in other systems such as liver (124), cauliflower (125), tobacco (126), etc., cytochrome-*c* is easily removed. Evidence is accumulating that the active form of cytochrome-*c* is a lipoprotein, both from the studies on cytochrome-*c* extraction from electron transport particles (117, 119), and from the requirement for phospholipid supplement to activate purified cytochrome-*a* (106) or cytochrome-*c* oxidase preparations (102, 118, 127, 128). The effects of lecithinases on liver, kidney, and brain mitochondria are consistent with this interpretation (129, 130) and studies on insect mitochondria by Wojtczak & Wojtczak (131), as well as corn mitochondria by Kahn & Hanson (68), indicate that endogenous lecithinases are present in mitochondria.

In some instances, therefore, the binding of proteins in mitochondria can be accomplished by formation of proteolipid or lipoprotein structures. The release of materials such as cytochrome-*c* or ATPase (129) from mitochondria would then depend on the action of endogenous or exogenous phospholipase or on the presence of activators (e.g., calcium) (8, 117) for the hydrolytic enzymes. Lehninger & Remmert (132) showed enzymic release of an uncoupling factor that is most likely a fatty acid.

Functional Lipid Molecules

Coenzyme *Q* is apparently present in plant mitochondria (133) and may be presumed to function in electron transport as shown for animal mitochondria (3, 134). However, evidence for this function is meager so far.

Solvent extraction procedures have been immensely useful in studying coenzyme *Q* function (134, 135). The same type of process was successfully used by Lynch & French (136) to extract lipids from chloroplasts and show restoration of activities when the lipid was re-added. A specific requirement for plastoquinone has been demonstrated by Bishop (137) in the chloroplast system. Tocopherol and other lipid materials have also been functionally implicated in rat liver mitochondria by Nason and co-workers (138, 139). Certain of the restorative effects of tocopherol and other lipids are undoubtedly related to a release of solvent inhibition in one way or another, as shown by Pollard & Bieri (140). Others have also shown reversal of solvent inhibition by added lipids (141, 142). On the other hand, Corwin & Schwarz (143) in a study of respiratory decline in vitamin E-deficient rat liver found no effect of vitamin E deficiency on DPNH—cytochrome-*c*—reductase, but they did find a decline of succinoxidase activity when DPN was present and attributed this to an accumulation of oxalacetate. An alternative interpretation of vitamin E function in the respiratory systems involves its antioxidant function (140, 144). The fact remains that tocopherol is present in animal mitochondria although the chance for a direct role in electron transport is lessened, as Deul *et al.* (141) point out by stating that there is no evidence for oxidation-reduction change. This fact does not exclude tocopherol function in phosphorylation (145). It might be well to ask if the tocopherol in plants is localized in mitochondria.

Not all lipids that restore activity in solvent-extracted mitochondria act nonspecifically to reverse solvent inhibition. Ambe & Crane (135) have shown that the restorative effects of coenzyme *Q* for succinic—cytochrome-*c*—reductase activity are specific to the quinone moiety. Certain other lipid factors (146) have also been found to be rather specific in their function, and induction of a requirement correlates with an appearance in the extracting solvent. It would seem that destruction of a lipoprotein unit within the mitochondrial membrane can lead to successive extractions of lipid materials originally responsible (by concerted action) for the function of the unit.

Factors in Phosphorylation

The fragmentation approach to the mechanism of oxidative phosphorylation has not produced dramatic results. It has been possible by certain treatments to obtain sub-mitochondrial fragments that will carry out oxidative phosphorylation. Lehninger *et al.* (147) have used digitonin treatment of rat liver mitochondria for this purpose while Green and co-workers (148, 149) have used high phosphate concentration or sonic treatment to disrupt mitochondria and yet retain phosphorylative capacity.

Certain factors have also been extracted from mitochondria that are required to recouple phosphorylation. Linnane (150) and Pullman *et al.* (151)

have both obtained a protein fraction that is necessary to couple phosphorylation to electron transport. The identity of the coupling factor remains unknown. McMurray & Lardy (152) have shown a requirement for coenzyme-*A* for phosphorylation in liver mitochondria. On the other side of the picture, Remmert & Lehninger (153) have reported a "loosening" factor that releases respiration from dependence on ADP in otherwise tightly coupled mitochondria without acting as an uncoupling agent when ADP is present.

Greater success with fragmentation studies is seen in the demonstration by Brodie and co-workers (154, 155, 156) that vitamin K_1 is required for oxidative phosphorylation in a bacterial system. Light-inactivation of oxidative phosphorylation in animal mitochondria also has been reversed by addition of vitamin K_1 (157 to 160), but there is no evidence as yet that vitamin K_1 is destroyed in mitochondria by the treatment or that vitamin K_1 is even present in mitochondria. Light-inactivation of adenosine triphosphatase activity of mitochondria has also been reported by Low *et al.* (161) and Beyer (162). The effect of light in this instance has been attributed to destruction of flavin (161). It would seem that the time has come to find out exactly which compounds in mitochondria are destroyed by light and which are involved in each of the inhibition effects.

Other Enzymes of Mitochondria

A large group of enzymes have been reported in mitochondria, especially from liver (1): the fatty acid oxidizing system, choline oxidase, α-glycerophosphate dehydrogenase, and the enzymes involved in lecithin synthesis and special protein synthesis. Previous reviews have discussed animal (1) and plant (9) mitochondria with respect to various activities. Further systematic study of the enzymes of plant mitochondria continues. Pierpoint (78, 81) has continued his studies of the enzymes of tobacco leaf mitochondria. Cheniae & Evans (163) have also found a nitrate reductase associated with mitochondrial material from soybean nodules. Bone (164) has described ornithine and citrulline synthesizing systems in mung bean seedling mitochondria as well as glutamic dehydrogenase (165). Spencer (166) was able to show ethylene production in mitochondria from ripening tomatoes. The activity reported in the mitochondria was no greater than that in intact fruit so evidence of localization of the ethylene producing system in mitochondria is not conclusive. Some of these functions are obviously important in the oxidation of respiratory substrates whereas others may be involved in the synthesis and breakdown of mitochondrial structure as shown by incorporation of choline into mitochondrial phospholipids (167).

Composition of Mitochondria

Our understanding of mitochondrial functions increases as we learn more about the molecular composition of mitochondria. The overall composition indicates a basic lipoprotein unit with most mitochondria consisting of 30 to 40 per cent lipid and 60 to 70 per cent protein (8, 122). Trace elements and

vitamins are also present [primarily for their catalyic potential (168)] but perhaps, as in the case of nonheme iron, they are structurally important also (101). Composition of mitochondrial lipids needs more study. At least 70 to 80 per cent of the lipid fraction is phospholipid (8, 169). These phospholipids are rather unique in their high level of unsaturated fatty acids (especially polyethenoid) (170) and their large proportion of plasmalogen (171, 172). The bulk of the remaining lipids in animal mitochondria are neutral fats and cholesterol (172). No full recovery of mitochondrial lipid has been achieved yet, so an uncertainty remains about possible unknown material. It is also intriguing to consider whether certain plant sterols are specifically associated with mitochondria.

Analysis of lipid composition in fragments of animal mitochondria has shown that lipids are not qualitatively nor quantitatively the same in all parts of the mitochondrion (7, 169, 173). This uneven distribution has led to the proposal that mitochondrial lipids are contained in discrete packets or lipoprotein units in the mitochondria and that each lipoprotein unit is associated with a certain catalytic function (3, 7). Further evidence for discrete functional lipoprotein units comes from detergent-induced fragmentation of beef heart mitochondria and the isolation of lipoproteins or lipid packets. Cytochromes-*c* and -c_1, diaphorase, and coenzyme *Q* have been isolated in functional lipoprotein or lipid units from heart mitochondria (3). It can be assumed that other members of the electron transport chain will be associated with other lipoproteins or phospholipid packets.

Certain other lipid materials are present in smaller amounts appropriate to a catalytic function. These include coenzyme *Q*, tocopherol, and possibly carotenoids. Some reports (174) state that vitamin K is a mitochondrial material whereas others do not indicate significant amounts (8, 133). In both plant and animal mitochondria other materials exist that show absorption spectra not relatable to known mitochondrial constituents. Since any compound with absorption in the ultraviolet or visible region must be considered of potential interest for the electron transport function, it is obvious that these unknown materials represent a large gap in our understanding of mitochondria.

Nucleic acid has also been reported in mitochondria although the amount is variable (cf. Table II). The presence of nucleic acid is consistent with the demonstrated synthesis of proteins in mitochondria. It would perhaps be easier to conceive of a synthesis of mitochondrial proteins in an ordered sequence *in situ* to account for the consistent and ordered structure of mitochondria rather than a localization of proteins, after synthesis in the cytoplasm, onto lipid surfaces. Interpretation of nucleic acid function in this way would indicate a higher proportionate nucleic acid content in young mitochondria than in old. Indication of a role for nucleic acid in mitochondrial function has also been presented by Pinchot's (175) studies of phosphorylation in bacteria and Hansen's studies on the effects of ribonuclease (89).

Analysis for discrete and unique molecules may be extremely useful in

TABLE II

RIBONUCLEIC ACID CONTENT OF PLANT MITOCHONDRIA

Source	RNA Content (μgP/mg. protein N)	Reference
Pea seedlings	29	Stafford (197)
Wheat roots	9	Martin & Morton (198)
Silver beet	8	Martin & Morton (199)
Castor bean endosperm	26	Akazawa & Beevers (200)
Wheat roots	25*	Lindblad (70)
Peanut cotyledons	[(0.164(E_{260})/mg. N)]	Kmetec & Newcomb (201)
Corn scutellum	19 μg RNA/mg. dry wt.	Hanson *et al.* (61)
Rat liver	17 μg/mg protein	Reis *et al.* (65)

* Estimated from dry weight value.

studies on the distribution of mitochondria or in the localization of enzyme functions. More of this is needed in correlation with enzyme studies because of the problems of inhibition and activation in enzyme assays. In other words, mitochondria may not always oxidize succinate especially if an inhibitor like malonate or catechol is around, but we hope they will always contain coenzyme *Q*. Likewise the distribution of copper in the cell should be consistent with assays for ascorbic acid oxidase, phenol oxidase, and cytochrome oxidase activities. If not, certain facets of the enzymatic activities in a given tissue remain to be explored, or other copper-containing proteins must be considered.

PURIFICATION

A technical problem that deserves much further study is the improvement of methods for purifying plant mitochondria. Plant cells seem to be blessed with a multitude of particulate or lipoprotein structures primarily defined by cytological studies (176, 177, 178). The diverse particles are also obvious in electron micrographs of plant mitochondrial preparations (61). Some of the problems encountered in the determination of the cytochromes of plant mitochondria may derive from the presence of other particulates. Lysosomes, described as a possible part of the electron-dense granule fraction from liver homogenates by Novikoff, Beaufay and deDuve (28), have been separated from liver as well as carotenoid granules from beef heart. The presence of both plastoquinone and coenzyme *Q* in a mitochondrial type of preparation from cauliflower buds (125, 179, 180) points to a mixture of mitochondria and proplastids in the preparation. Precise determination of the chemistry and enzymological properties of plant mitochondria will be greatly advanced by better methods of separation and purification.

The methods of electrophoresis and gradient centrifugation applied by James & Das to leaf mitochondria should be useful in other material (181).

New techniques such as special filtration systems (182) and the use of non-aqueous media (183, 184) will provide approaches to the purification problem. Even techniques that do not yield physiologically intact mitochondria may be useful if they provide better definition in the assay or isolation of mitochondrial components.

Precise sedimentation studies as carried out with liver particles by Beaufay *et al.* (185) should give better definition of enzymatic functions of the particles in plant cells. Forti *et al.* (186) have reported separation of particles in pea stems by differential centrifugation.

CONCLUSION

The last few years have been a time of change in the study of mitochondria. Prior efforts were concentrated on the definition of mitochondria as the seat of respiratory function and in studies of what might be called "mitochondrial physiology." Methods for isolating mitochondria in the best possible condition for studies of oxidative phosphorylation and citric cycle function were devised. Indirect methods for the study of phosphorylation and electron transport were developed based on inhibition effects, spectrophotometric studies, and isotope exchange. Now we are in the era of "mitochondrial chemistry." The cytochromes, flavoproteins, and other enzymes are extracted and purified. Trace elements are determined. The lipid fraction is undergoing analysis and the individual units are isolated. From functional studies of electron transport and from the association of parts within the whole it has become apparent that the mitochondrion is made up of subunits. First we have the parts defined by electron microscope study, the membranes and the internal matrix. Functional study of these parts indicates that the membrane is the heart of function since this is the site of electron transport and oxidative phosphorylation. When the membrane is broken up by detergents or solvents it dissociates into smaller, discrete, and chemically distinct units of lipid and protein, each with some part of the original enzymic function. Lipid has become more significant with the demonstration of electron transport function for lipids like coenzyme *Q* and vitamin K. Thus we are engaged in the study of biphasic lipid and aqueous systems organized into units called lipoproteins or proteolipids. We must still identify all the molecules in the units, and above all we must find how the molecules are arranged in the structure. When this is known we may expect to know how mitochondria function and why they are influenced in certain ways by external agents. Consideration of mitochondria as a lipoprotein membrane system emphasizes the basic importance of the lipid phase in cellular function. Life may be made possible by the stabilizing influence of a lipid phase in an aqueous medium. The biphasic system provides for the separation of reactants and an orderly sequence of reactions not possible in a uniphasic system.

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STRUCTURE AND PHYSIOLOGY OF CELL WALLS[1,2,3]

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The cell wall is important biologically for two reasons. First, as one of the main differentiating cellular elements it determines the morphology and to some degree, the functions of the cell. Second, in forming the limiting envelope of the cell, the wall may be directly involved in regulating cell expansion. These two aspects of the wall are reflections of its well-known dual organization (93): a discontinuous microfibrillar phase embedded in a continuous phase formed of an amorphous matrix. The microfibrils are relatively inert and rigid and their number and organization are largely responsible for the form and structural characteristics of the wall. Matrix materials also contribute to structure but are generally considered more reactive than the microfibrils and have been assumed to control wall rigidity, thereby influencing growth.

In this review, our principal aim is to discuss the structure and physiological responses of the wall in terms of these two phases with particular reference to differentiation and growth of the cell. The discussion will be confined almost entirely to primary walls, that is, the walls of enlarging cells (133, 166) of higher plants. Since we attempt to consider wall properties in molecular terms, the first part of the review is devoted to a brief survey of the constituents of walls and their biosynthesis.

Chemical Composition of Walls

Polysaccharide components.—The microfibrillar component of walls usually consists of cellulose or chitin while the amorphous matrix is composed predominantly of polysaccharides which can be classified (170) simply as hemicelluloses and pectic substances. Native cellulose appears to be similar in both primary and secondary walls of higher plants but almost all knowledge of non-cellulosic matrix substances is derived from studies of mature secondary walls and is useful here only to indicate possible components of primary walls.

Cellulose occurs naturally as microfibrils 100 to 200 A wide, within which long parallel polymers of predominantly β-1,4 linked D-glucose residues form a well-ordered core surrounded and interrupted by regions of less order (57, 82, 124, 126, 130, 166). A model for the ordered arrangement of molecules in native cellulose was proposed by Meyer & Misch (104) in which neighbour-

[1] The survey of literature pertaining to this review was concluded September 1960.

[2] The following abbreviations are used: PME (pectin methyl esterase); PP (pyrophosphate); UDP (uridine diphosphate); UDPG (uridine diphosphate glucose); UTP (uridine triphosphate).

[3] Issued as N.R.C. No. 6150.

ing molecules run in opposite directions, i.e., are anti-parallel, although the evidence for this is not conclusive. This model, which was later modified by Frey-Wyssling (58) to provide better agreement with the observed behaviour of microfibrils, has not been supplanted although recent studies have found it inadequate (55).

Celluloses from different sources differ in microfibril width (11, 162), degree of crystallinity (82), and in the traces of non-glucose residues found associated with them (1). There is no evident correlation between these features (12), but Preston (124) and Roelofsen (133) suggest that non-glucose residues are concentrated in the less-ordered regions of the microfibril. It has not been proved, however, that such residues are covalently linked to cellulose (10) and it is possible that they are merely adsorbed since customary extraction procedures are known to be incapable of separating artificial mixtures of cellulose and hemicellulose (150).

Chitin, a polymer of N-acetylglucosamine, replaces cellulose as the microfibrillar component in many fungal walls (9, 59, 133). In the well-founded model for chitin proposed by Carlström (34), the molecules are antiparallel. Instances of other microfibrillar polysaccharides have been reported, notably among the algae (124), but they do not appear to occur generally and their significance is not clear at present.

The hemicelluloses, which have been discussed recently by Aspinall (10) and Whistler & Sannella (170), are a heterogeneous group of compounds containing D-xylose, L-arabinose, D-galactose, D-glucose, D-mannose, and D-glucuronic acid residues. Generally one or two residues predominate in a backbone chain, allowing four relatively distinct groups of compounds to be recognized: xylans, mannans and glucomannans, galactans and arabogalactans, and glucans. The xylans and arabogalactans show considerable branching, branches of the former usually containing mixtures of several residues, while glucomannans are relatively unbranched. Pure galactans, mannans, and glucans are rare in higher plants (10) but the latter two are common in fungi (9, 115). Preliminary analyses of primary walls of oat coleoptiles (18, 131) indicate the existence of several hemicellulose fractions and the presence of all of the above component residues, suggesting that these walls contain several general types of hemicelluloses.

The pectic substances are basically straight chain polymers of α-1,4 linked D-galacturonic acid residues which are usually partially esterified with methyl groups. Molecular weights ranging from 25,000 to 360,000 have been reported (114). Much pectin of walls occurs as relatively insoluble protopectin, which may represent very long polygalacturonic acid chains (24) or molecules extensively cross linked (114). Protopectin of oat coleoptiles contains a relatively low percentage of methyl esters (88), thereby increasing the possibility of carboxyl interaction. Henglein (81) presents an extensive discussion of all aspects of pectic substances.

Quantitative studies.—Analyses of a number of primary walls are summarized in Table I. Most of the data were obtained by selective extraction

TABLE I

SUMMARY OF QUANTITATIVE CHEMICAL ANALYSES OF PRIMARY WALLS

[Values represent percentages (rounded off) of total dry wall material.]

Object	Reference	Cellulose	Hemicelluloses	Pectic Substances	Protein	Lipid
Corn coleoptile— 9 mm.	(174)	38	46	10	—	7
Corn coleoptile—32 mm.	(174)	33	34	10	—	23
Corn coleoptile—50 mm.	(174)	35	30	13	—	21
Corn coleoptile	(107)	28	22	—	30	7
Corn coleoptile	(95)	27	—	8	2.5–5	—
Corn mesocotyl	(133)	30	50		3	2
Oat coleoptile	(158)	42	38	8	12	—
Oat coleoptile	(18)	25	>51	<1	10	4
Oat coleoptile	(88)	—	—	5	—	—
Pea stem	(38)	26	40	20	13	1
Young wheat leaves	(30)	30	11	22	—	—
Young wheat roots	(30)	38	15	16	—	—
Sunflower epicotyl	(78)	37	4	53	—	—
Sunflower hypocotyl	(78)	38	9	46	—	—
Tobacco pith	(172)	—	—	5–8	—	—
Potato tuber	(33)	29	—	16	—	—
Young cotton hair	(159)	50	—	10	13	8
Young cotton hair	(169)	—	—	5–6	—	—
Young cotton hair	(98)	33	20	23	—	24
Pine cambium	(6)	20–25	—	10–20	—	—
Black locust cambium	(8)	—	—	<13	—	—

coupled with gravimetric or relatively non-specific chemical analyses. Because of the reasonably distinct solubility properties of cellulose, the values given for this substance are probably fairly accurate and indicate that it usually forms only 25 to 35 per cent of dry walls. The figures for the preponderant matrix substances are less reliable since chromatographic analyses (18, 92, 131) reveal considerable cross contamination between fractions usually designated as hemicellulose and pectin. In oat coleoptiles at least, hemicelluloses are the most abundant matrix materials and only small amounts of pectic substances are present. Most earlier analyses probably underestimate hemicelluloses and overestimate pectic substances.

The actual amount of pectin in oat coleoptiles is in doubt. Using two different extraction procedures, Bishop *et al.* (18) found less than 1 per cent galacturonic acid, but characterized it by several direct methods. On the other hand, with the pectinase-carbazole method Albersheim & Bonner (5) obtained 3.5 to 4 per cent and Jansen *et al.* (88) 5 per cent anhydrouronic

acid. Galston & Purves (62) argued that the latter method, being gentler, is more likely to give accurate quantitative values. Kivilaan *et al.* (95) also obtained less galacturonic acid from corn coleoptile walls by chemical hydrolysis and chromatography than Albersheim found, presumably by pectinase-carbazole, and suggested hydrolytic destruction to account for the discrepancy. The destruction argument is reasonable but before the pectinase-carbazole values are taken as final this method should receive more critical evaluation. We have found that, even after extensive purification, commercial pectinase preparations readily hydrolyse hemicelluloses and may liberate sugars which could upset quantitative uronic acid determination.

Table I shows that an appreciable amount of nitrogen, presumably from protein, accompanies primary walls. Probably much of this is due to plasmodesmata and cytoplasmic contamination (18), but the 2.5 to 5 per cent protein remaining in well-washed preparations of isolated walls (95) may represent enzymes bound in the wall. Pectin methylesterase has been shown to be bound to cell walls (28) outside the cell membrane (68); and in the light of recent evidence that cell wall deposition can occur remote from the cytoplasm (144), it is probable that other enzymes involved in cell wall metabolism may be found in walls. Ginzburg (64) also suggested that cells may be cemented together by a complex of protein and non-cellulosic substances. His evidence for this is indirect and derived from cells of the extreme tips of roots where the walls are tenuous and contain abundant plasmodesmata (168). The discussion of the middle lamella, below, suggests that a proteinaceous cement in elongating cells is unlikely.

Lipid constituents are commonly found associated with primary walls (Table I) and are probably derived mainly from cuticle. It appears, however, that some waxes also occur within walls although their nature and function is obscure (133). Electron microscopy of cells fixed with osmic acid (144) and permanganate (168) has not consistently revealed any structure that could represent organized lipids within primary walls.

The above discussion concerns dry wall material, but it is well known that the native primary wall is highly hydrated. A figure of over 90 per cent water in young walls is often quoted (59) but Roelofsen (133) disagreed with this and obtained a more reasonable value of 60 per cent water for isolated walls of corn coleoptiles.

Composition of the middle lamella.—The paucity of pectic substances in walls as typical as those of coleoptile parenchyma raises the question of the nature of the middle lamella. Kerr & Bailey (94) defined this structure as the amorphous intercellular material between adjacent primary walls and electron microscope observations on sections of extracted walls confirm the existence of such a cellulose-free region (15, 143). It has also been widely accepted that the middle lamella is principally composed of pectates, but the evidence needs re-examination as it rests mainly on non-specific extraction and staining procedures (23).

Even if all of the 1 to 5 per cent polygalacturonic acid of coleoptile walls

were localized in one layer between cells it probably could not be resolved in the light microscope. A systematic electron microscope study of the middle lamella has not been performed, but no discrete intercellular layer can be discerned consistently in either unshadowed (95a, 168) or shadowed sections (Setterfield & Bayley, unpublished data). In the latter type of section, the texture of the matrix appears uniform throughout the wall. Concerning extraction of the middle lamella, Ginzburg (64) has pointed out that conditions needed to separate root-tip cells with EDTA (ethylenediamine tetraacetic acid) are far more drastic than expected if simple chelation of calcium and solubilization of pectic acid were involved. Similarly, we have consistently observed both with coleoptiles and with the elongation zone of roots, that treatments required to separate cells readily also thoroughly extract matrix substances from the entire wall revealing clean microfibrils in the electron microscope (142). These considerations suggest that the middle lamella may merely be a region of the polysaccharide matrix where cellulose is absent, rather than a region of pectate localization. On the basis of electron microscope examination of walls of pea root tips extracted with EDTA and stained with uranium, Klein & Ginzburg (95a) also concluded that there was no specialized cementing layer between walls. Specific extractions, perhaps by purified enzymes, coupled with electron microscopy should clarify this situation.

Chemical interactions and wall structure.—The physical properties of walls must depend largely on interactions between the various wall substances. If wall structure is likened to reinforced concrete with microfibrils corresponding to reinforcing steel, it is clear that, for strength, interactions must occur between microfibrils, between matrix substances, and between microfibrils and matrix. Furthermore, in the primary wall such interactions must be labile enough to permit controlled wall expansion and deposition.

The nature of such interactions is largely unknown but several possibilities exist. Carboxyl groups of pectic substances and hemicelluloses containing uronic acid residues possess an obvious capacity for reaction through ionic bridges and ester bonds. Such bonds are commonly invoked in theories of wall structure (17, 81, 128, 155) but since some growing walls contain only small amounts of uronic acid residues this type of interaction may be of limited importance (18, 131). It is possible but unproven that pectic substances are strategically dispersed through the wall and act as key bridging agents with the hemicelluloses. Certainly the common occurrence of insoluble protopectin and the presence of arabans and galactans in pectic preparations (114) suggests that pectic substances are closely linked to other components of the matrix.

Of the matrix polysaccharides, the preponderant hemicelluloses probably make the main contribution to primary wall structure. These substances possess less obvious capacity for covalent bonding than the pectic substances and they probably interact largely through hydrogen bonds and van der Waal's forces. In discussing cellulose structure it was pointed out that hemi-

celluloses bind readily to cellulose. Difficulties in purifying individual hemicelluloses (10) suggest that they also interact with each other. The branched structure of some hemicelluloses would increase the chance of molecular entanglement and interaction (166). Recently Jensen (90) suggested that hemicelluloses in walls hold cellulose under tension since their removal with alkali caused 30 per cent shrinkage of 10 μ longitudinal sections of onion roots. It seems unlikely that the relatively short segments of microfibrils present in such sections would be under tension and the shrinkage was probably due to mercerization (82) of the cellulose. We have obtained shrinkage of sections with alkali extraction but it is not apparent when hemicelluloses are removed with enzymes.

Biosynthesis of Wall Polysaccharides

It will become evident in later sections that knowledge of the biosynthesis of wall components is basic to an understanding of both the structural development and metabolic responses of walls. Unfortunately, although intermediary carbohydrate metabolism is extensively elaborated the nature of the reactions leading to polysaccharides of walls is largely unknown. This section summarizes available evidence on the subject and discusses some of the outstanding problems. Recent reviews of some aspects of the subject are given by Hassid *et al.* (80), Neish (111), and Stone (153).

Cellulose.—Knowledge of cellulose synthesis in plants is derived solely from studies using isotopically labelled substrates and essentially consists of one well-established, somewhat unsurprising, fact; viz. the intact glucose carbon skeleton is used directly in some form or other for cellulose synthesis. Several workers administered specifically ^{14}C-labelled glucose to cotton bolls (70, 147) and wheat plants (27, 50) and found that it entered cellulose with little skeletal rearrangement. The small amount of rearrangement found by all workers except Greathouse (70) indicated some degradation and resynthesis of hexose by Embden-Meyerhof glycolytic reactions (110). Besides glucose, Neish and co-workers (7, 27, 109, 110) supplied wheat plants a large number of other specifically ^{14}C-labelled monosaccharides and related compounds and their results consistently indicated that glucose was the most efficient precursor of cellulose and that other compounds such as pentoses, sedoheptulose, and succinic acid were converted to hexose before entering cellulose.

The mechanism of glucose polymerization is unknown but sugar phosphates and nucleotides have been implicated. In particular Neish (110, 111) focused attention on uridine diphosphate glucose (UDPG) since known enzymatic reactions involving this compound could explain distribution of administered isotope into other cell wall polysaccharides (see below.) Furthermore, UDPG has been shown to be a substrate for enzymes producing a number of polymers containing glucose: glycogen (99), β-1,3 linked glucan (53), type III pneumococcal capsular polysaccharide (149), and hyaluronic acid (103). However, attempts to demonstrate an enzyme from plants which

will synthesize cellulose from UDPG have been unsuccessful (80) although Glaser & Brown (66) found an enzyme, chitin synthetase, in a particulate fraction of *Neurospora crassa*, which converted UDP-N-acetylglucosamine to chitin. The relative ease with which this enzyme was obtained, compared with the failure to demonstrate an analogous cellulose synthetase in higher plants, suggests that the systems for chitin and cellulose synthesis may be distinctly different despite similarity of the products.

Certain bacteria, notably *Acetobacter xylinum*, produce extracellular cellulose (83) as a "by-product," allowing study of cellulose synthesis without problems of cell wall deposition. Schramm *et al.* (137) investigated the carbon metabolism of this organism and showed that glucose was rapidly converted to gluconate and that a well-developed pentose-phosphate pathway was present by which 6-phosphogluconate was decarboxylated at carbon-1 and resynthesized to hexose phosphate. Results from supplying glucose, specifically labelled with ^{14}C in different positions, indicated that cellulose was synthesized from such hexose phosphate and that as in higher plants the intact hexose skeleton was used in the polymerization reaction. Greathouse (71) also administered specifically labelled glucose but although the results indicated direct utilization of the glucose skeleton in cellulose synthesis they were inconsistent with operation of the pentose-phosphate pathway. The reason for this discrepancy is not clear.

Several attempts have been made to obtain cellulose synthesis in cell-free preparations of *A. xylinum*. Although Gromet *et al.* (79) detected many other enzymatic activities, they could not demonstrate cell-free production of cellulose. Colvin (45) used an homogenate supplemented with glucose and ATP and by electron microscopy detected very low levels of microfibril production. Glaser (65) demonstrated a small amount of net cellulose synthesis using a particulate cell fraction, UDPG-^{14}C and cellodextrin primer. Recently, Klungsöyr (96) claimed to have enhanced cellulose synthesis through this reaction by adding excess UDP (uridine diphosphate) and cellodextrins to the homogenate. Unfortunately this author used alkali insolubility as the only criterion for cellulose and Colvin (45) has shown that amorphous non-cellulosic material insoluble in alkali may be obtained from *A. xylinum*. Greathouse (72) described a cell-free preparation from *A. xylinum* which, when incubated with glucose, ATP, and yeast extract, gave 15 per cent yield of cellulose from glucose. This yield is remarkable as the methods used for preparing the extract were not essentially different from those of other workers. Since the methods used to characterize the product as cellulose were not given these results must await independent confirmation. Finally, Colvin (46) reported that ethanol extracts of *A. xylinum* contained substances that formed microfibrils when water and a heat-labile catalyst from the culture medium were added. He could not detect cellobiose, related oligosaccharides, UDP, or UDPG in the extract, suggesting that an entirely new mechanism is involved in cellulose formation. It is too early to explain the differences between Glaser's and Colvin's results as neither obtained

more than trace amounts of cellulose, but as the former author notes, more than one pathway to cellulose may operate in this bacterium.

Aside from the biochemistry, formation of cellulose by *A. xylinum* has some peculiarities which deserve comment. Electron microscope studies (47, 48) indicate that the microfibrils grow at their tips without contact with the surface of the cells, a finding of considerable importance in relation to microfibril growth in walls. There are two difficulties with such a system: first, well-washed cells immediately form cellulose without the lag period expected if polymerizing enzyme must first be formed and liberated into the medium (83); second, if the immediate precursor of cellulose is a glucoside, it is improbable that the cells could lose large amounts of the aglycone after the glucose entered cellulose (153). The first objection would be satisfied if microfibrils were initiated on the bacterial surface and then separated from the cell, carrying polymerizing enzymes bound to their tips. If such is the case, gently prepared, short microfibrils should act as primers for cellulose synthesis in cell-free systems. The second problem is more difficult. Possibly much of the aglycone could be reabsorbed by the cells, and some form of surface diffusion along the microfibrils might reduce necessary external concentration of the precursor.

Roelofsen (133) discusses the additional problem in cellulose synthesis of the relation of simple polymer formation to microfibril organization. He concludes that glucosan chains and microfibrils are formed simultaneously by addition of individual glucose residues at the microfibril tip rather than by organization of pre-formed glucosan molecules into microfibrils. On the basis of the following evidence we would tentatively agree: (*a*) dispersed molecules of cellulose will not reform into native microfibrils (82, 133); (*b*) under the electron microscope the tips of growing microfibrils in *A. xylinum* cultures appear smooth and tapered, not frayed, and no evidence of an amorphous precursor is found (47, 48); (*c*) abundant oligoglucosides are not found in *A. xylinum* (46); and (*d*) microfibrils are formed readily by *A. xylinum* in stiff gels of carboxymethyl cellulose which would presumably interfere with migration of large cellodextrin molecules (48). However, simultaneous formation of the β-glucosan chain and microfibril presents an enzymatic problem (133). If glucosan chains in the microfibril run in both directions, i.e., antiparallel, both free carbon-1 and carbon-4 will be present at both ends of the microfibril and two enzymes would be required for growth. Alternatively, with a parallel arrangement of glucosan chains one enzyme could bring about growth at one end of the microfibril only. It is impossible to choose between these systems at present, other than to note that the antiparallel arrangement of molecules in chitin (34) favors the two enzyme hypothesis.

Non-cellulosic carbohydrates.—The only available information on synthesis of the non-cellulosic carbohydrates of walls concerns metabolism of the constituent monosaccharides. The utilization of many specifically ^{14}C-labelled precursors in a variety of plants has been studied: Neish and co-

workers (7, 27, 109, 110) followed incorporation from several hexoses and pentoses into xylan of wheat; Ginsburg & Hassid (63) analysed both xylan and araban of wheat following administration of labelled sugars; Seegmiller and co-workers administered specifically ^{14}C-labelled glucose and galactose to boysenberries (139) and strawberries (140), and analysed the uronic acid and arabinose of a pectin fraction; Loewus *et al.* (101) supplied ^{14}C-labelled glucuronate, glucuronolactone, and galactose, and analysed polygalacturonic acid of strawberries. The results from all of these studies are in general agreement and indicate that the hexose skeleton remains predominantly intact during formation of all of the 6-carbon components of wall polysaccharides. Further, little rearrangement of the first 5 carbon atoms occurs during the formation of xylose and arabinose from either hexoses or hexuronic acids. This result suggests that the pentoses are formed by oxidation and decarboxylation of carbon-6 of the hexoses (110, 111). In support of this Slater & Beevers (148) showed that in corn coleoptiles carbon-6 of exogenously supplied glucuronate and glucuronolactone was rapidly lost as CO_2. Feeding of pentoses indicates that arabinose can be used directly for pentosan formation, but in wheat and strawberry xylose must be converted to hexose before entering wall pentosan (7, 100, 109, 111).

The interconversions of labelled compounds in the above studies are consistent with the scheme in Figure 1, which is based on those of Neish (111) and Hassid *et al.* (80). The individual reactions are catalysed by the following

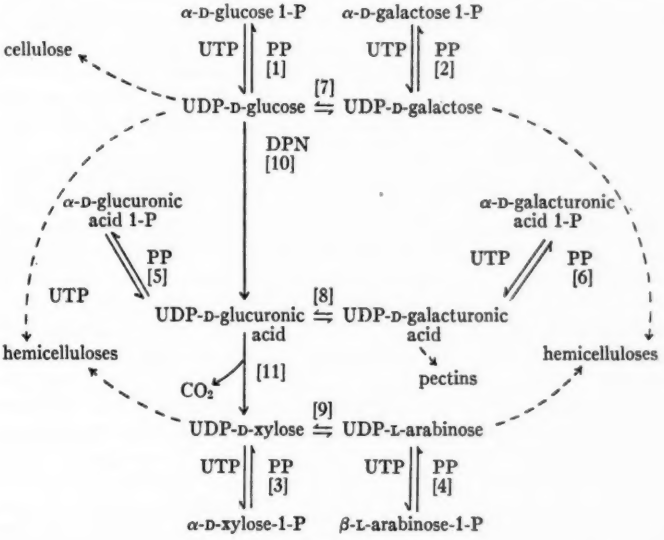

FIG. 1.

types of enzymes (80): [1 to 6] pyrophosphorylases; [7 to 9] C-4 epimerases; [10] dehydrogenase; [11] decarboxylase.

Extensive evidence for such a scheme in higher plants comes from studies by Hassid and co-workers (80) on mung bean seedlings. They isolated all of the sugar nucleotides shown in the diagram and demonstrated enzymes that catalysed all numbered reactions. In addition, the enzyme for reaction [10] has been isolated from pea seedlings (154) and for reaction [11] from asparagus, radish, and spinach (112). The apparent failure of exogenous xylose to enter directly the xylans of wheat (109) and strawberry (100) suggests that reaction [3] is not effective in these plants and that xylose is rapidly drawn into the pentose-phosphate pathway and partially converted to hexose phosphate (111). Apparently arabinose is readily utilized through reaction [4] but there is difficulty in explaining the conversion from the pyranose form used in reaction [4] to the furanose form found in hemicelluloses (80). UDP-mannose is not included in the scheme since it has not been reported, but guanosine diphosphate mannose has been found in yeast (32) where cell wall mannan is present (115). There is as yet no evidence for the direct utilization of the UDP derivatives in the reactions of polysaccharide formation shown by broken arrows in the diagram. Feingold *et al.* (54) described an enzyme from asparagus that utilized UDP-xylose to form short β-1,4 linked xylo-oligosaccharides but felt it was not responsible for xylan synthesis.

Some isotope experiments to determine the origin of methyl ester groups in pectin have been performed. They indicate that the methyl group of methionine may esterify pectic carboxyl groups in radish plants (136), in oat (120), and corn (41) coleoptiles, but not in corn mesocotyls (41). Formate, formaldehyde, the α-carbon of glycine, and the β-carbon of serine can also be utilized by radish (175). In oat seedlings methionine may also be converted to methionine sulphoxide and S-methyl-methionine, and these derivatives can donate methyl groups to pectin (135).

Arrangement of Microfibrils in Walls

The microfibrillar organization of primary walls has been thoroughly described in recent reviews by Frey-Wyssling (59), Roelofsen (133), and Wardrop (166) and will not be considered in detail here. Since in the past few years important similarities in the organization of very diverse primary walls have become evident, in this and the following section we will emphasize general aspects of primary wall structure, noting only the more significant exceptions.

Simple multinet structure.—The cellulose skeleton in the walls of some young isodiametric cells, such as the apical initials of onion roots (138), is a rather disorganized mesh of microfibrils, although in young parenchyma cells of oat coleoptiles a net transverse orientation of microfibrils can be discerned (19). As elongation proceeds, a pattern becomes established in thin primary walls in which the majority of microfibrils show wide angular dispersion about a mean orientation transverse to the cell axis—the "tubular

texture" of Frey-Wyssling (56). Closer examination of such walls reveals a difference in orientation of microfibrils on the inner and outer surfaces. Although the microfibrils are interwoven throughout the thickness of the wall and are not arranged in layers, nevertheless, on the inner surface they are transversely oriented in a fairly regular fashion whereas towards the outside they show greater dispersion and an increasing tendency to longitudinal orientation. Because of the model of wall growth proposed by Roelofsen & Houwink (134) and discussed in the next section we shall call this type of arrangement "simple multinet structure." In this structure transverse microfibrils predominate, thereby accounting for the negative birefringence observed in the walls of many elongating cells (22, 133).

Simple multinet structure was first demonstrated clearly in the walls of the sporangiophores of *Phycomyces*, staminal hairs of *Tradescantia*, cotton hairs, and hairs from the fruits of *Ceiba* and *Asclepias* (85, 134, 159). The same type of structure has since been described in the walls of parenchyma cells from oat coleoptile (19, 142, 164), onion root (142, 164), celery petiole (143), and bean stem (145, 146), and in young collenchyma cells of celery (15). Scott *et al.* (138) did not describe their results on onion roots in terms of multinet structure but their observations appear consistent with such an interpretation. Although the wall of the internodal cell of the alga *Nitella* is considerably thicker than the walls just mentioned, this too was found to possess simple multinet structure (77). All of these cells elongate over most of their length, but similar structure has also been observed in the primary walls of differentiating tracheids of conifers (165) where growth is localized at the cell tips.

These observations indicate that simple multinet structure with predominantly transverse microfibrils is of general occurrence in thin primary walls. A possible exception is in root hairs (85) which grow only at their tip where the wall contains a thin mesh of microfibrils with little or no preferred orientation. This meshwork persists below the tip on the outside of the wall and a secondary wall of longitudinal microfibrils is apposited on the inside. The wall structure of a number of algae is also quite distinct from that of higher plants and has been given separate consideration recently by Preston (124) and Roelofsen (133).

Longitudinal arrangements of microfibrils.—In the walls of many parenchyma cells (19, 143, 167) and of conifer tracheids (165), axial ribs composed of longitudinally oriented microfibrils occur between regions of simple multinet structure. The ribs of coleoptile parenchyma are present in very young cells and persist throughout elongation. In the electron microscope face views of parenchyma walls clearly show the ribs on the outer surface (19, 143, 167) and reveal microfibrils running from longitudinal orientation in the ribs to transverse in the inter-rib regions. Transverse sections of such walls disclose two types of rib (143); "integral ribs" in which microfibrils throughout the thickness of the wall are longitudinally oriented, and "outer ribs" that consist of a separate layer of longitudinally oriented microfibrils

outside the microfibrils forming the simple multinet structure. Such ribs were previously regarded as secondary thickenings; but the distinction between these structures and secondary wall is clear in oat coleoptiles and onion roots from sections of fully-elongated parenchyma cells in which layers of closely aligned, near-longitudinal microfibrils representing a secondary wall can be distinguished on the inner surface of the primary wall (143). The orientation of microfibrils in these secondary walls probably varies (133), as Wardrop & Cronshaw (167) report finding them at angles of between 30 and 60° to the cell axis in oat coleoptile parenchyma.

Another, more striking, instance of longitudinal microfibrils in primary walls is found in collenchyma cells. Preston and co-workers discovered longitudinally oriented cellulose in collenchyma walls of *Heracleum sphondyllium* (102) and *Petasites vulgaris* (127) with the polarizing microscope, and they were the first to describe these as "thickened primary" walls. Electron microscope observations of collenchyma in celery petioles (15) revealed that young walls had a simple multinet structure while in progressively older cells distinct layers of longitudinally oriented microfibrils appeared at several points around the circumference of the cell. The number and extent of the layers increased with cell age and in mature cells the wall contained a large number of layers running continuously around the lumen. Thickened primary walls containing longitudinal microfibrils are also present in outer epidermal cells (13), guard cells (141), and sub-epidermal cells (142) of oat coleoptiles, in epidermal and cortical cells of bean stems (145, 146), and in the epidermal wall of onion roots (142). In young oat coleoptiles the outer epidermal wall consists of 10 to 15 layers of microfibrils oriented longitudinally in the outer layers but more nearly transversely in the inner. As elongation proceeds the number of layers increases to about 25 while the microfibrillar orientation becomes predominantly longitudinal in all layers (13). Similar layers have been observed in epidermal cells of *Hyacinthus* (106) and *Philodendron* (20) but it is not certain to what extent these represent primary and secondary walls.

The above studies indicate that organized groups of longitudinally oriented microfibrils are a common feature of primary wall structure.

A generalized structural pattern for primary walls.—Superficially, thickened primary walls containing numerous layers of longitudinally oriented microfibrils seem quite distinct from thin parenchyma walls with their predominant simple multinet structure. From the preceding discussion, however, it is clear that such a distinction is arbitrary and the arrangements of microfibrils in most primary walls can be regarded as variations of the same basic organization (143). This organization consists of two distinct microfibrillar components: transverse microfibrils which as a result of cell elongation form a simple multinet structure (see below), and organized layers of longitudinal microfibrils. Different types of primary wall reflect variations in the relative amounts of these two components. Thus in the extreme case of hair cells (85, 134, 159) and internodal cells of *Nitella* (77) the longitudinal

component is absent and the entire microfibril arrangement is simple multinet. Ordinary parenchyma cells (19, 143, 167) are intermediate in having both components present while collenchyma cells (15, 102) have a predominant longitudinal component. Epidermal cells (13) are a special case in which the longitudinal component predominates in the outer wall but is absent from the radial walls. The cortex of the elongation zone of bean stems (145, 146) shows that this concept is not completely artificial. In this tissue, there is a gradual transition from extensively multilayered walls in the epidermis and outer collenchyma through walls with progressively fewer layers to typical parenchyma walls deep in the cortex. This transition is achieved by a change in the proportion of the two microfibril components which are deposited.

One clear exception to the above discussion should be mentioned. Sterling & Spit (152) studied walls of developing fibres in asparagus and their observations showed that the inner surface of the wall consisted of a system of thin layers of microfibrils in which the orientation relative to the cell axis alternated at an angle of 55 to 60° for most cells. In young fibres the outside of the wall showed predominantly transverse microfibrils but in older fibres the outermost microfibrils were oriented longitudinally. Unfortunately this study is open to the criticism (133) that, because of the preparative procedure employed, the inner and outer surfaces of the walls could not be conclusively identified in the electron microscope. Such cells might profitably be reinvestigated with direct sectioning techniques.

Origin and Development of Wall Structure

Sites of wall extension and deposition.—Marker experiments on the epidermal cells of oat coleoptiles (37) have established that these cells grow uniformly over their whole length. Studies on the distribution of primary pit fields indicate the same type of growth in elongating parenchyma cells of oat coleoptiles (163) and *Elodea* stems (173). Autoradiography of coleoptile and onion root parenchyma cells supplied ^{14}C-labelled sugars (19, 142, 164) showed that incorporation took place uniformly over the entire cell surface. Furthermore the conclusion that incorporation was not localized in small areas such as primary pit fields was later made more secure by high-resolution autoradiography with tritium (144). Control studies by Ordin *et al.* (119) indicate that the isotope incorporation in these autoradiograph studies represents new wall synthesis rather than turnover. These experiments support the generalization that cells of axial organs undergoing primary growth enlarge over their whole length and that new wall is deposited over the entire surface. A number of other cells apparently undergo localized growth, e.g., sporangiophores, root hairs, pollen tubes, cambial initials, tracheids, and phloem and xylem fibres [see Roelofsen (133) and Wardrop (166)], but there appears no reason why these examples should not be regarded as special cases of uniform growth.

Two attempts to establish the site of wall deposition in relation to wall

depth have been made. Using counting methods with tritium, Green (74) showed that deposition is confined to the inner surface of walls of internodal cells of *Nitella*. These cells elongate uniformly along their length (73). On the other hand, we have shown by tritium autoradiography that deposition of both cellulose and non-cellulosic substances occurs throughout the thickness of outer epidermal walls of oat coleoptiles (144). It is interesting that these two thickened walls represent opposite extremes of growing wall structure, since *Nitella* walls have only the simple multinet arrangement of microfibrils (77) while the epidermal walls are composed entirely of multilayered longitudinal microfibrils (13). As will become clear below, the different modes of deposition are compatible with these differences in structure.

Deposition throughout wall thickness raises the question of the relation of cytoplasmic structure to wall deposition. In electron microscope studies of well-preserved cells we found no evidence either of penetration of walls by cytoplasm except at restricted plasmodesmata, or of any consistent organized non-carbohydrate structure within the walls (144). Examination of numerous electron micrographs of plant cells published by other workers (168) supports this conclusion. Dense bodies described in epidermal walls (15) are artifacts (144), and the structures reported by Newcomb & Siegesmund (113) and discussed by Galston & Purves (62) probably also fall into this category. The absence of cytoplasm in walls that are being added to throughout their thickness led to the suggestion that formation of wall polysaccharides may occur remote from the cytoplasmic membrane (144). Such a proposal is not new as it was made by Beer (16) to explain growth of pollen walls.

Theories of wall growth based on structure.—Detailed examination of walls by electron microscopy has led to the formulation of several new theories of wall growth which have been discussed in detail recently (59, 133, 166). Of these, protoplasmic tip growth (56, 105) and mosaic growth (56, 151) appear to have been based on misinterpretations of the appearance of wall fragments in the electron microscope. Neither these theories nor the ideas of plasma papillae (26), islands of synthesis (129), or localized synthesis at pit fields (163), are supported by the evidence discussed above.

The most attractive theory concerning structural development of thin primary walls is that of multinet growth proposed by Roelofsen & Houwink (134), and because of its value we shall examine it in some detail. The theory proposes that during elongation the cell continually lays down microfibrils on the inner surface of the wall in a direction approaching transverse, i.e., in a helix of almost zero pitch. Extension of the wall causes these transverse microfibrils to be drawn out so as to increase the pitch of the helix and produce a gradation of microfibril orientations from transverse on the inner surface to approaching longitudinal on the outer, as described in the previous sections. The system, as the name implies, is somewhat analogous to a series of nets drawn out longitudinally to different extents although the wall is not in fact composed of distinct layers. Since the cell diameter does not decrease as the microfibril helices are drawn out, the theory requires that microfibrils

slip relative to each other. Indication that such slippage occurs comes from the fact that epidermal walls containing numerous longitudinal microfibrils elongate uniformly along their length (37).

This simple multinet theory provides an entirely satisfactory explanation of the origin of the simple multinet structure described in the previous section and would appear applicable to all elongating walls in which transverse microfibrils are deposited. The behaviour of microfibrils in walls that are rapidly expanding without being reinforced is not clear, however. Bonner found that no wall deposition occurred in oat coleoptiles expanding in the cold (21) but under the polarizing microscope the net transverse orientation of the cellulose in the parenchyma walls remained unaltered (22). On the other hand, Wardrop & Cronshaw (167) found in the electron microscope that, as expected on simple multinet theory, microfibrils in the walls of similar cells showed marked reorientation. The reason for this discrepancy is not clear, and it is evident that re-examination of this system and of rapidly elongating *Tradescantia* staminal hairs and filaments of grass stamens, previously studied only in the polarizing microscope (60, 86), would be useful.

The simple multinet theory does not explain the presence of organized layers of longitudinal microfibrils in primary walls. Wardrop & Cronshaw (167) suggested that longitudinal ribs in parenchyma walls might result during elongation from a greater reorientation of transverse microfibrils at the cell corners where plasmodesmata are absent. It seems unlikely that such a mechanism alone could account for prominent ribs since thinning must accompany reorientation and furthermore, ribs have been observed in very young parenchyma cells (19, 167) where little reorientation resulting from elongation could have occurred. Bolliger (20) recently suggested that epidermal cell walls of leaves are formed by multinet growth but the electron microscope evidence he presented is inconclusive. It is clear that the multinet theory could not explain the regular outer ribs found in parenchyma (143) and multilayers of longitudinal microfibrils in collenchyma walls where there is no evidence of gradual transition in orientation (15). Moreover, we have recently shown that extensive deposition of longitudinal microfibrils occurred in cells of bean stems where elongation is much reduced so that little reorientation could occur (145, 146). Obviously to account for the longitudinal component of primary walls a theory must explain the deposition of microfibrils in this orientation rather than relying on reorientation after deposition.

Roelofsen has recently sought to elaborate the multinet theory to account for all types of primary wall structure (132, 133). The central tenet of his argument is that stress in walls determines microfibril orientation, a view based essentially on two facts.

(*a*) When an amorphous substance, e.g., a gel, is stressed the molecules become oriented parallel to the stress.

(*b*) In the walls of many cylindrical cells the microfibrils are preponderantly transverse and it is known that in a cylindrical vessel under pressure

the greater stress in the wall is transverse, being twice the longitudinal stress (36). From this it is argued that microfibrils are always deposited and kept oriented in the direction of maximum stress and can only be reoriented by a change in the direction of maximum stress. Thus in simple multinet structure microfibrils are deposited in the direction of the greater transverse stress and then, since the inner layer of microfibrils takes up the bulk of this stress, the outer microfibrils are free to be reoriented to the direction of the longitudinal stress which now predominates. The presence of abundant longitudinal microfibrils in some walls is explained on the basis that these walls are under longitudinal tension generated by surrounding cells and since this tension exceeds the normal transverse stress, longitudinal microfibrils are deposited.

This elaboration of the multinet theory is open to considerable criticism. As originally pointed out by Preston (123) it appears that the roles of stress and strain are not clearly recognized. In a gel it is strain which orients the molecules, not the stress producing strain. Cylindrical cells undergo very little radial enlargement and thus there is very little transverse strain in the walls that could produce transverse orientation of microfibrils. On the other hand, longitudinal strain in the wall due to elongation will tend to reorient transverse microfibrils after deposition in all primary walls, producing the recognized simple multinet structure. However, even if a causal relationship between direction of maximum stress and direction of microfibril deposition is granted, the hypothesis seems untenable. There is no evidence, either experimental or theoretical, for the complex system of tensions between different cells which Roelofsen invokes to explain deposition of longitudinally oriented microfibrils. The theory would require that in a celery petiole, for example, the directions of maximum stress in the walls of young parenchyma and collenchyma cells differ by 90° and that the tension be so regulated that collenchyma would form in regularly spaced bundles. Despite Roelofsen's persuasive arguments, we are unable to visualize such a mechanism. The multinet theory must therefore remain primarily a description of events following deposition rather than an explanation of the control of microfibril deposition.

In our opinion the direction of microfibril deposition is closely controlled by the protoplasm as part of genetically determined differentiation. The mechanism of this control is unknown, but its existence is clearly indicated by the highly specific, complex arrangements of microfibrils found in secondary walls and in walls of algae (123, 133). Among the latter, for example, Green (76) recently showed that complex circular arrangements of microfibrils occur in growing walls of *Bryopsis* at regularly spaced regions destined to become branches. In different species of higher plants it is also clear that occurrence and form of primary walls such as those of collenchyma and guard cells (52) is basically part of the overall pattern of differentiation and not simply a result of non-specific physical forces such as stress or turgor pressure.

It is difficult to conceive how ordered layers of longitudinal microfibrils in

outer ribs of parenchyma and thickened primary walls could initially be formed remote from and yet under the control of the cytoplasm. Once formed, they are probably maintained during cell elongation by deposition of new microfibrils throughout the wall (144), the arrangement of which is presumably controlled by the existing structure.

One approach to understanding the way in which the protoplasm controls microfibril orientation is by experimentally modifying wall structure. In a young growing bean stem such modifications can be achieved by horizontal constraint (26), and on the basis of electron microscope observations they were interpreted as follows (145, 146). On the lower side of the horizontal stem cortical cells are induced to expand radially and they respond by depositing more transverse microfibrils. In contrast, cells are not expanding radially on the upper side of the stem but are under considerable bending stress and respond by depositing stiffening layers of longitudinal microfibrils. The latter response is probably similar to that found in celery petioles subjected to bending stresses where the collenchyma walls are thicker (160, 161). These results do not, however, give any information on the direct mechanisms controlling deposition since the nature of cellular response to stress is unknown. Furthermore, it must be re-emphasized that the stresses merely influence an inherent capacity to respond since, for example, only localized groups of cells in celery petioles are capable of forming collenchymatous thickenings.

Control of Growth by Cell Walls

Apart from the morphogenetic significance of the primary wall just described, its possible role as an active mediator of cell enlargement may be important. Opinion on this latter subject has varied, but interest has been reawakened by a number of recent studies (62, 157). Although most of the evidence is indirect, by implication it mainly concerns the continuous phase of the wall, the polysaccharide matrix (93).

Mechanisms of cell expansion.—Three obvious physiological processes occur during normal growth of the cell; water enters, new wall material is deposited, and the wall increases in area. Growth might conceivably be controlled by direct regulation of any of these reactions. Studies on water metabolism have been reviewed by Galston & Purves (62) and by Thimann (157) and the evidence indicates that osmosis rather than "active" water uptake plays the major role in water movement. Experiments with growth regulators further indicate that control of growth by direct regulation of osmotic properties of cells is unlikely and although such properties generate turgor pressure, which serves as a driving force in growth, the mechanisms of control reside elsewhere in the cell, perhaps in the cell wall. Attention is thus directed to the latter two growth processes, deposition and expansion of walls.

Heyn (84) outlined three possible mechanisms of wall expansion: (*a*) increase in surface area by active synthesis of new wall; (*b*) elastic extension of the wall by turgor pressure, made permanent by deposition of new wall

substance; (*c*) direct plastic stretching of the wall by turgor pressure. In the first case control of growth is exercised by direct regulation of mechanisms of wall synthesis while in the other two schemes, controlled variation of physical properties of the wall could regulate growth.

The first mechanism, active wall growth, has been discarded on the basis that it requires strict stoichiometry between deposition and elongation and that uncoupling of the processes can be demonstrated (84). For example, Preston & Clark (125) showed that in intact oat coleoptiles longer than 1 cm. weight of wall per unit length decreased steadily during elongation, resulting in about 30 per cent "thinning" of the walls at cessation of growth. Similar situations where rate of expansion exceeds rate of deposition have been noted in isolated segments of oat coleoptile (14, 31, 158) and pea stem (38). Using optical techniques Green (75) found an initial rise followed by a sharp drop in mass per unit area of *Nitella* internodal cell walls in early stages of growth, and then a slow increase during the main portion of elongation. However, although such partial non-correspondence of deposition and expansion suggests that deposition is not the immediate cause of growth it is not conclusive proof of this. Active growth on the inside of the wall, for example, could promote elongation while the outer regions are passively thinned.

Several other lines of evidence, however, indicate that active wall deposition is not the basic driving mechanism in cell elongation. Bonner (21) found small amounts of elongation without deposition of wall in coleoptile segments at 2°C. Several workers (44, 97, 156) showed that the regulatory action of auxin can be separated in time from the act of growth. The presence of simple multinet structure, discussed earlier, indicates that in thin parenchyma walls intussusception of new microfibrils does not occur throughout the wall but rather deposition of cellulose is primarily by apposition (133). Finally, there is abundant evidence to indicate that walls become more plastic in response to growth regulators. As a result of considerations such as these, the theory of growth by wall deposition has been largely dismissed in favor of theories involving stretching of the wall. However, as will be discussed below, growth and deposition are not fortuitously related processes. Furthermore, direct evidence on mechanisms of wall growth is scanty and it is possible that enlargement by active wall growth may occur in some cells (29).

Growth through wall plasticization.—During the 1930's Heyn (84) performed extensive experiments to demonstrate the importance of wall plasticity in growth. In particular he treated coleoptile segments with auxin under conditions of much reduced growth and noted that plasticity of the walls, as revealed by bending under weight, varied in relation to the capacity for elongation. He also studied the elastic properties of the wall and found that they were not correlated with capacity for expansion but depended on the growth history of the segment. From these and other experiments Heyn concluded that control of growth is achieved by varying the plasticity of the wall and that plastic extension, produced by turgor pressure, is the primary mechanism of cell growth.

Recently several workers have repeated and extended Heyn's observations. Tagawa & Bonner (155) confirmed that auxin produced a relatively rapid increase in plasticity of coleoptiles as measured by plastic bending and observed that calcium and magnesium ions, which retard elongation, caused a stiffening of the wall while potassium ions, which stimulate elongation, increased plasticity. Adamson & Adamson (2), using bending techniques, also found increased plasticity of coleoptile walls with auxin and a decrease with calcium chloride. They also noted that although anaerobiosis and low temperature increased rigidity of the tissue auxin still increased wall flexibility under these conditions. Cleland (39) refined Heyn's experiments by applying auxin in the presence of 0.28 *M* mannitol, which osmotically prevented elongation, and concluded with Heyn that growth stimulation by auxin was directly related to increased plasticity of the wall. Objections to determinations of wall plasticity by bending measurements have been met by Preston & Hepton (128) who measured physical extensibility by applying a force in line with the axis of the coleoptile. By this method it was demonstrated that auxin caused a noticeable increase in extensibility of walls even when growth was limited by mannitol or absence of water. In contrast to other workers (39, 84), Preston & Hepton concluded that elastic properties of the wall may be important in growth. However, the coleoptiles used in many of their experiments elongated without added sugar and may have shown changes in elasticity due to thinning of walls (17, 84).

Additional support for Heyn's plasticity theory comes from studies in which action of growth substance is separated in time from the period of growth. Thimann (156) retarded the growth of potato discs with mannitol and found that on subsequent removal of the mannitol the rate of cell expansion exceeded that of controls. A somewhat more definitive experiment was performed by Kobayashi *et al.* (97) who incubated oat coleoptile segments with and without auxin in 0.25 *M* lactose, which inhibited growth, and then transferred all segments to water without auxin. Segments which had received auxin with the lactose grew significantly more in water than ones treated with lactose alone. This experiment was further refined by Cleland & Bonner (44) who gave an aerobic pretreatment with auxin in a concentration of mannitol which inhibited growth and then allowed expansion to occur in water under argon, a condition which normally inhibits auxin stimulation of growth. They observed a significant increase of elongation under argon caused by auxin in the pretreatment. All of these results are readily interpreted on the basis that auxin produces a plasticization of the wall which persists, and then results in increased elongation when turgor pressure is restored.

The evidence therefore strongly supports Heyn's notion that plasticity of the cell wall is an important factor in cell growth and that it can serve as the basis of at least one growth regulating mechanism. However, there are some ambiguities concerning the role of metabolism in the process. At low temperature, increases in extension (21, 84) and flexibility (2) produced by

auxin have both been reported. Preston & Hepton (128) however could find no increase in physical extensibility at low temperature and Cleland & Bonner (44) noted that elongation under argon following auxin treatment in mannitol was also prevented by low temperature. Furthermore, auxin-induced growth is prevented by anaerobiosis (44) and all auxin effects on plasticity and extension are abolished by cyanide (39) and other metabolic inhibitors (38, 44). Yet auxin stimulation of plasticity under anaerobic conditions is reported (2). The inhibitor evidence would definitely suggest that plasticization of the wall must be a metabolic process.

Chemical nature of wall plasticization.—Bennet-Clark (17) in reporting growth inhibition by divalent ions, particularly calcium, suggested that they might reduce wall plasticity by increasing ionic binding between pectic carboxyl groups [a theory also advanced by Carlier & Buffel (33)] and that growth stimulation by ethylenediamine tetraacetic acid was due to its decreasing such cross-linking by chelating calcium. Interaction of divalent ions with pectin was also advanced by Tagawa & Bonner (155) to explain decreased plasticity of coleoptile walls treated with calcium and magnesium. Additional evidence for involving pectin in wall plasticity came from studies of pectin methyl esterase (PME). Bryan & Newcomb (28) found a large apparent increase in the amount of this enzyme in tobacco pith cells stimulated to enlarge by auxin. Similarly, Yoda (176) noted that inhibition and stimulation of growth of pea stem segments by various substances was closely paralleled by changes in PME activity in tissue homogenates. In a number of *in vitro* (67) and *in vivo* (69) studies Glasziou showed that auxin can increase binding of PME to walls and proposed that such binding prevented de-esterification of pectin, thereby decreasing the amount of calcium bridging. This mechanism has been invoked to explain auxin effects on wall plasticity at low temperatures and in anaerobic conditions (2). Other authors (49, 51) have taken the effects of commercial preparations of pectinase on growth of roots to indicate that pectin is involved in wall rigidity.

Although such indirect evidence is suggestive, the hypothesis that calcium-pectin interaction controls wall plasticity is not supported by recent studies. The low amount of pectin found by Bishop *et al.* (18) in oat coleoptile, if accurate, seriously restricts such a mechanism. Furthermore, the calcium content of primary walls appears to be very low. In wheat coleoptile walls Carr & Ng (35) detected only 0.1 per cent calcium on a dry weight basis, a figure corresponding to about 1 per cent calcium pectate. Similarly, Jansen *et al.* (88) found only enough calcium in oat coleoptile walls to form the salt of one-fifth of the non-esterified anhydrouronic acid residues which in turn represented only 3 per cent of the dry wall. More directly, Cleland (42) used a sensitive assay with calcium^{45} and showed that in both corn mesocotyls and oat coleoptiles auxin had no effect on the amount or distribution of calcium in growing walls. Earlier, Carr & Ng (35) were unable to find any removal of calcium by ethylenediamine tetraacetic acid under conditions where growth stimulation would be obtained. The general importance of

pectin methyl esterase in growth is doubtful since Jansen *et al.* (89) found no evidence of auxin-mediated binding or stimulation of this enzyme in oat coleoptiles. It is also difficult to see how the binding mechanism could allow auxin to produce a rapid increase in growth once considerable calcium cross-linking had occurred. Experiments using commercial pectinase to influence growth have been justifiably criticized by Jackson (87) on the basis that such enzyme preparations are very impure.

A more direct test of the pectin hypothesis in growth might be expected from comparative chemical analyses of walls grown with and without auxin. Several such analyses exist but the picture is confused. Using fairly specific methods for polygalacturonic acid, Wilson & Skoog (172) found a 50 to 100 per cent increase in the concentration of pectic substances in tobacco pith segments treated with auxin, whether or not growth resulted. Carlier & Buffel (33) noted a somewhat smaller increase in concentration of pectic materials in potato tuber discs supplied with auxin. On the other hand, Jansen *et al.* (88) claimed that auxin caused no change in the pectic composition of oat coleoptile walls. Using less specific methods, Burström (30) also concluded that relative concentrations of wall materials do not change significantly in normal growth of roots.

Several studies on patterns of ^{14}C incorporation have been performed, but again the results are equivocal. Boroughs & Bonner (25) supplied ^{14}C-labelled acetate and sucrose to coleoptiles and found that auxin increased incorporation of isotope into the fraction soluble in 4 per cent NaOH (hemicelluloses) and decreased incorporation into other wall fractions. Surprisingly, although auxin stimulated growth it caused a slightly decreased total incorporation of ^{14}C into the wall from both substrates. Wightman & Neish (171) noted increased incorporation into both hemicelluloses and cellulose of oat coleoptiles and pea stem segments due to auxin treatment. There was, however, no difference in the relative incorporation into the two fractions. In excised wheat roots Perlis & Nance (122) used auxin as a growth inhibitor and obtained an increased relative incorporation of ^{14}C from pyruvate and acetate into cellulose. As they pointed out, this was probably related to synthesis of secondary wall and in fact multilayered secondary walls are common in the parenchyma of roots (142). Nance (108) also supplied acetate-^{14}C to pea stem segments and noted decreased isotope incorporation into non-cellulosic wall materials due to auxin. This result was apparently due mainly to alterations in intermediary metabolism rather than immediate effects on synthesis of cell wall. Ordin *et al.* (119) administered glucose-^{14}C to coleoptiles prevented from elongating by mannitol and found little difference in incorporation due to auxin. Recently, however, Albersheim & Bonner (5) using labelled glucose have shown that auxin stimulates the rate of synthesis of pectic material soluble in cold water. They suggest that this stimulation could be a basis for wall plasticization, but since the fraction forms only about 0.2 per cent of the total wall material and is presumably not very closely bound in the wall its significance in growth seems questionable.

One reasonably clear cut, although again quantitatively small, metabolic response of pectin during growth involves methyl esterification. Ordin *et al.* (119, 120) supplied methyl-^{14}C-methionine to coleoptiles and found an auxin-increased rate of incorporation of ^{14}C into methyl ester of pectic substances soluble in hot water. Subsequently, Jansen *et al.* (88) showed that this increased incorporation was due to increased turnover, not net synthesis of methyl ester. However, Cleland (41) was unable to demonstrate that auxin stimulated incorporation of ^{14}C from methyl-labelled methionine into pectic substances of corn mesocotyls and suggested that either auxin-stimulated methylation of pectin is not a general mechanism or a different methyl donor is used in mesocotyls. Cleland (43) also found in oat coleoptiles that, although ethionine rapidly prevented incorporation of methyl groups from methionine and at the same time reduced elasticity of the wall, inhibition of growth was not directly related to these processes.

Taking the evidence as a whole, no consistent major change in the metabolism of pectic substances in relation to growth has been demonstrated. Thus, although the hypothesis that pectic substances determine plasticity of the wall is attractive theoretically, it remains unsubstantiated. Unfortunately, the studies that failed to demonstrate a role for pectin also failed to show an obvious growth response of other wall substances, suggesting that the basis of plasticity of the wall may reside in qualitative, rather than quantitative, changes in the preponderant and complex hemicelluloses. That slight structural changes are capable of changing the properties of these compounds is indicated by studies such as those of Perlin (121), who showed that the solubility of xylans is directly related to the frequency of arabinose side chains.

Relations between cell elongation and wall deposition.—Although plastic stretching appears to be important in growth, wall deposition normally accompanies cell elongation and even if, as noted above, the two processes do not always remain perfectly in step it is impossible to separate them for long periods. Thus, coleoptile segments may elongate 15 or 20 per cent without wall deposition at low temperature (21) or without oxygen (39), but sustained growth under these conditions is impossible. Limiting substrates for wall synthesis will also limit growth (31) and galactose may inhibit growth of coleoptiles by direct inhibition of cellulose synthesis (118). General metabolic inhibitors will of course inhibit both processes (38) but simple osmotic inhibition of elongation also prevents wall deposition (14, 116). Growth inhibition by lack of auxin is accompanied by decreased wall synthesis (14, 38). These facts lead to the conclusion that although wall deposition may not be the direct cause of elongation, the two processes are closely interrelated and essential for each other during continued growth.

Dependence of wall deposition on extension might be visualized as some process by which stretching of the wall frees active surface areas required for addition of new materials. Such a mechanism could explain reduction of wall deposition in the absence of auxin but the situation is more complex in the

case of osmotic inhibition of growth. By manipulating external concentrations of mannitol and diffusible salts, Ordin (117) obtained evidence that synthesis of wall in oat coleoptile segments depends directly on turgor pressure rather than on elongation. A similar proposal was made by Adamson & Adamson (3) to explain results from Jerusalem artichoke. Ordin (116) also found that synthesis of different wall fractions shows a variable sensitivity to turgor although the main trend is for reduced synthesis with reduced turgor. In this connection it might be noted that, without entering the cell, mannitol could exert direct effects on polymerization reactions occurring outside the plasma membrane.

The alternative relation, dependence of wall elongation on deposition, might be explained on the basis that a given amount of wall material is only capable of a certain amount of stretching by turgor, and for continued expansion to occur, new material capable of being stretched must be added. Auxin would mediate the stretching process but could only function in the presence of wall material which was not fully stretched. The plastic material would presumably be a component of the matrix and would be added by intussusception throughout the wall while the transverse microfibrils were apposited on the inner surface. Such a concept would not be in entire agreement with the simple multinet theory in its present form, but the site of deposition of matrix substances in parenchyma walls is as yet unknown. If these substances are added only by apposition they must be capable of indefinite stretching and a more complex feedback mechanism would be required for expansion to be limited by wall synthesis.

To this discussion should be added the ideas of Busse (31) that both plastic substance and material capable of stiffening walls are added during deposition and that the rate of elongation depends on a balance between the two. He has presented evidence that stimulation of coleoptile growth by cobalt results from an inhibition of synthesis of the stiffening material. Auxin-independent stiffening of coleoptile walls has also been noted by other workers (4, 44) and Cleland (40) has invoked two types of bonds, auxin sensitive and auxin insensitive, to explain the fact that elongation of coleoptiles supplied with auxin shows a sharp change in rate at an external mannitol concentration of about 0.12 *M*. The stiffening materials would presumably be a component of the matrix since structural studies indicate that the microfibrils, although reasonably rigid themselves, are able to slip past each other in the matrix of growing walls. Once stiffening of the matrix stopped growth, further reinforcement could be achieved by deposition of secondary wall.

As noted by Busse (31), considerations of this type are quite speculative and can serve only as working hypotheses. However, they have several consequences. First, growth may be experimentally regulated by interference with at least four processes: water uptake, plasticization by auxin, synthesis of plastic materials, and synthesis of stiffening materials. Second, a change in

the composition of the matrix substances should be detectable as the proportions of stiffening and plastic materials change. Jermyn & Isherwood (92) have shown that quite complex changes can occur in walls of parenchyma cells during ripening of fruit and it seems reasonable to expect that changes of a similar nature take place during the course of growth. Recent cytochemical analyses of walls by Jensen & Ashton (91) and Fuller (61) suggest that fairly complex changes occur in root meristems but more detailed chemical study of matrix substances will be necessary to test these ideas fully. Here, studies of lower plant forms which appear to have walls of relatively simple composition (9, 115) may be useful.

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MINOR MINERAL NUTRIENTS[1,2]

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Several well documented surveys within the last five years have covered various aspects of the trace element nutrition of plants (1 to 10). The purpose of this review is not to catalogue the work done on trace metals during the last few years but rather to attempt to show how trace metals are concerned in some of the major metabolic pathways of plant metabolism. The term plant is used here in its wider and indeed correct sense to include bacteria, fungi, algae, and higher forms. It is noteworthy that the recent work on the role of trace metals in the metabolism of higher plants was often preceded by work with microorganisms.

Essentiality of Mineral Nutrients

At the present time the following elements are known to be required for the growth of plants: N, P, K, Mg, Ca, S, Fe, Cu, Zn, Mn, Mo, B, Cl, Na, Co, and V. Not all of these elements are universally required by plants but all of them have been found to be essential for some plant. The mineral nutrients are usually subdivided into two main groups: (*a*) major element or macronutrient and (*b*) minor element, micronutrient, trace element, or oligo-element and, as the names suggest, they reflect a large or a small requirement for the elements by plants (11). Although this is a convenient classification it is arbitrary and has obvious limitations. Thus Ca and S are essential in large amounts to higher plants whereas microorganisms usually have small requirements for them. Even in the micronutrient category, comprising Fe, Cu, Zn, Mn, Mo, B, Cl, Na, Co, and V, there is a very wide range of requirement. Thus the need for Fe may well be a thousand times that for Mo which in turn may be greater than for Co. There is some justification, therefore, for an ultra-micro group of biologically important metals, e.g., Mo, Co, and V, to which other elements may be added in due course when they are shown to be necessary for growth.

[1] The survey of literature pertaining to this review was concluded in November 1960.

[2] The following abbreviations are used: CDTA (cyclohexane trans 1,2-diamino tetraacetic acid); Chel-138 or EDDHA [aromatic phenolic polyamino-carboxylic acid (full identity not yet released)]; DPNH (reduced diphosphopyridine nucleotide); DTPA (di-ethylenetriamine petaacetate); EDTA (ethylenediamine tetraacetic acid); FAD (flavin-adenine-dinucleotide); $FADH_2$ (reduced flavin-adenine-dinucleotide); FMN (flavin mononucleotide); $FMNH_2$ (reduced flavin mononucleotide); HEEDTA (hydroxyethyl ethylenediaminediacetic acid); TPNH (reduced triphosphopyridine nucleotide); YADH (yeast alcohol dehydrogenase).

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There is also the problem of the differential requirements for trace metals in plants and even in the same plant under different conditions of growth. Thus boron is universally required by higher plants but the evidence that it is essential for bacteria and fungi is not conclusive. The recent demonstration of a cobalt requirement for legumes when fixing atmospheric nitrogen but not when grown on combined nitrogen (12, 13, 14) suggests that the micronutrient is required by the nodule bacteria and not the legume plants *per se*. Arnon (15) showed that vanadium is essential for the alga, *Scenedesmus obliquus*, and Bertrand (16) claimed a similar requirement for *Aspergillus niger*, but there are no reports that the micronutrient is necessary for higher plants. Arnon's suggestion that vanadium would be found to be essential for green plants is not yet substantiated. This may, of course, be due to great technical difficulties involved in growing higher plants in culture solutions deficient in the microelement. On the other hand, Hewitt points out that this forecast is not necessarily justified either on logical or on evolutionary grounds (9). Further experimental work will be required to arbitrate between these two hypothetical viewpoints. The molybdenum requirement for *Scenedesmus obliquus* and *Chlorella pyrenoidosa* is abolished when they are grown on ammonia or urea instead of nitrate (17, 18), whereas in fungi (19) and higher plants (20) there is still a small requirement for the element when these are grown on ammonium only.

Arnon suggested that three criteria should be used to establish whether or not a mineral element is essential for the growth of plants (21) and these have been widely accepted. His now classical proposals for an essential element are: (*a*) the organism cannot complete its life cycle without it, (*b*) its action must be specific and cannot be replaced by any other, (*c*) its effect on the plant must be direct. As pointed out elsewhere (6, 22), concept (*b*) may be too rigid in the light of recent work. Two examples illustrate this point. Molybdenum is required for N_2 fixation by *Azotobacter* but in some species vanadium has a sparing action. Both trace metals occur in certain soil habitats in equal proportions (23) so that either could be utilized. In fact Keeler & Varner (24) showed that vanadium concentrated in the same protein fractions as did molybdenum in *Azotobacter*. Chlorine is necessary for growth in higher plants but other halides, e.g., bromine, can substitute for it, although usually at a higher concentration (25). It is reasonable to assume, however, that chlorine is the most abundant naturally occurring halide in the soil and often in the air, especially near coastal areas. According to Arnon's definition, neither vanadium in *Azotobacter* nor chlorine in higher plants can be accepted as essential elements. To overcome this difficulty it has been suggested that a broader concept might fit in better with recent work on the role of trace metals in plant metabolism. The term "functional or metabolism nutrient" can be used to include any mineral element that functions in plant metabolism irrespective of whether or not its action is specific. This would also avoid problems such as an element being required only when a certain

substrate is present, e.g., *Scenedesmus* requiring molybdenum only when grown on nitrate nitrogen.

Two methods have been employed to determine the essentiality of trace metals for growth: (*a*) rigorous removal of metals from the basal culture solutions (26, 27, 28), and (*b*) demonstration of a role for them in plant metabolism (usually in association with essential enzyme systems). As pointed out elsewhere (6) the two methods are often used in a complementary way. Advances in the first method depend on finding more efficient techniques for removing metals from culture solutions, and the use of a wide range of organisms, in particular marine microflora which are likely to exhibit unusual trace metal requirements. It is clear that further progress along these lines will necessitate (for higher plants) the use of specially contructed growth chambers containing sterile air from which dust particles have been rigorously removed and fitted with automatic irrigation devices. The second method (*b*) is equally difficult but it is likely that this approach may be the most fruitful in this era of metabolic studies. It may be that unusual metals will be found in association with plant proteins as in animal tissues. Thus Margoshes & Vallee recorded a cadmium containing protein in liver tissue (29) but its function, if any, has not been determined.

Nitrogen Metabolism

This area of metabolism is chosen for first consideration since in the last few years minor elements have been found to be very important functional constituents in the enzyme systems involved.

Nitrogen fixation.—It is well established that molybdenum and iron are required for N_2 fixation in free-living and nodule bacteria. Esposito & Wilson have summarised the data (30) and have shown that marked differences exist among *Azotobacter* species in regard to the need for trace metals for N_2 fixation. Thus, *Azotobacter vinelandii* had moderate requirements for iron and calcium and a rather low demand for molybdenum. Nicholas (31) and Bové, Bové & Arnon (32) confirmed that molybdenum is essential for N_2 fixation although its replacement by vanadium may vary with the species. Takahashi & Nason (33) and Keeler & Varner (34) showed that tungstate is a competitive inhibitor of molybdate in nitrogen fixation and Keeler & Varner (24) demonstrated the incorporation of tungstate into the same protein fraction as molybdenum. Nicholas (31) showed that iron and molybdenum restricted N_2 fixation in *Azotobacter* and in *Clostridium pasteurianum*. Carnahan & Castle (35) demonstrated a larger iron requirement in *Clostridium pasteurianum* when it was fixing N_2 than when it was grown with ammonia. They suggest that the active site in the nitrogen-fixing enzyme could be a variable-valent metal ion with the power to transfer electrons to N_2 gas thereby reducing it to ammonia. In their opinion the metal could be either iron or molybdenum. Winfield (36) has previously proposed that fixation occurs by hydrogenation at the site of the ion iron complex in hydrogenase. Roberts

(37) has made an admirable review of the theoretical aspects of the mechanism of nitrogen fixation in relation to the metals. It remains to be seen whether the function of the metals in N_2 fixation will be resolved now that cell-free extracts have been prepared from *Clostridium pasteurianum* [Carnahan *et al.* (38)], from blue green algae and *Rhodosporillium rubrum* [the Wisconsin group (39)]from *Azotobacter vinelandii* [Nicholas & Fisher (40, 41)], and by Arnon *et al.* from *Chromatium* (42). Voets (43) and Bershova (44) also determined iron and molybdenum requirements in *Azotobacter* while Gerretsen & Hoop showed that 2 p.p.m. boron is essential for N_2 fixation in *A. chroococcum* (45). Gribanov (46) claimed that copper and manganese, at 5 mg. each/l., stimulated fixation in the same bacterium. Fogg & Wolfe (18) studied the effect of molybdenum on N_2 fixation by blue green algae and when grown on nitrate. In the presence of ammonia there was no requirement for the micronutrient.

Hydrogenase enzyme has been known to occur in most N_2 fixing organisms but there is a difference of opinion in regard to its metal constituent. Shug *et al.* (47) had earlier claimed molybdenum to be required for hydrogenase action in *Clostridium pasteurianum*, but other workers have shown that this enzyme depends on iron for its activity in this and in other bacteria (48).

It is well established that iron and molybdenum are required for N_2 fixation in root nodules. Thus, haemoglobin, first demonstrated by Kubo (49) in nodules, was found to vary in quantity with the condition of the nodule. Virtanen *et al.* (50) and Jordan & Garrard (51) reported a relation between the amounts of haemoglobin and the efficiency of fixation. Levin, Funk & Tandler (52) showed that vitamin B_{12} is present in greater amounts in the pink nodules of lucerne than in the white ones. As a result of careful work by Ahmed & Evans (12, 13), cobalt has now been established as an essential element for soybeans when fixing N_2 but no requirement was evident when plants were grown on combined nitrogen. Hallsworth, Wilson & Greenwood (14) have demonstrated that copper and cobalt stimulate N_2 fixation in subterranean clover. Holm-Hansen, Gerloff & Skoog (53) had earlier implicated cobalt in the N_2 fixation mechanism in blue green algae. It is of interest that Hewitt & Bond (54) have demonstrated a molybdenum requirement for N_2 fixation in non-legumes, e.g., *Casuarina* and *Alnus*.

Nitrate.—Earlier work on the molybdenum requirement in microorganisms and green plants assimilating nitrate nitrogen has been confirmed, as has the fact that the micronutrient is a functional constituent of the nitrate reductase enzyme (1 to 6; 8, 9).

In the last few years much work has been done on the role of metals in the dissimilatory nitrate reductase process including investigations on *E. coli* and denitrifying bacteria. It is now clear that iron, usually as a cytochrome, is an additional requirement when nitrates are dissimilated by microorganisms (55, 56) and even by higher plants (57). There is convincing evidence that molybdenum is required for the terminal step of all nitrate reductases so far

examined. In fact, terminal nitrate reductase appears to contain molybdenum and the nitrate reductases vary only in the type of electron carrier system preceding the terminal molybdenum-nitrate step.

Itagaki & Taniguchi (58) grew *E. coli* under the same conditions as did Nicholas & Nason (59) and found that 30 per cent of the enzyme activity was in the soluble fraction after high speed centrifugation. The enzyme was inhibited by cyanide and azide but not by CO, thus indicating a metal requirement; it was not identified. The following scheme (Fig. 1), based on inhibitor studies and their (58) past experience with the organism, was put forward to explain the mechanism of action

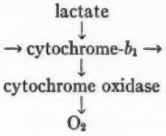

FIG. 1

Taniguchi & Itagaki (60) solubilized and purified another nitrate reductase from *E. coli* grown anaerobically and showed that cytochrome-b_1 was in the sequence of electron transfer to nitrate. Although they suggested a metal component, since cyanide, azide, and *o*-phenanthroline inhibited the enzyme, it was not identified. The inhibition of nitrate reductase by CO, first reported by Sato & Egami (61), was not confirmed by Joklik (62); Taniguchi (63) now reports this to be a variable effect. Erkama *et al.* (64) showed that iron deficiency depressed the dissimilatory nitrate reductase in *E. coli* but there was no effect on cell yield. Appleby & Morton (65) demonstrated that cytochrome-*b* is associated with lactic dehydrogenase in yeast and this may well acount for some of the recent results of the Japanese school who frequently use lactate as a hydrogen donor. The evidence for cytochrome-*b* being involved in nitrate reductase is sometimes based on inhibition by 2-heptyl-4 hydroxyquinoline-N-oxide but this is not a specific inhibitor of cytochrome-*b* [Kogut & Lightbown (66)]. It seems likely that the terminal step in nitrate reduction does not involve a cytochrome system but is dependent on molybdenum, as suggested earlier by Nicholas & Nason (59). Indeed, Taniguchi & Itagaki (67) have now solubilized a dissimilatory nitrate reductase from *E. coli* and have shown it to contain one atom of bound molybdenum and about 40 atoms of iron per molecule of enzyme. They suggest the following sequence (Fig. 2) of electron transfer from cytochrome-b_1 to explain their results.

They also state, without giving evidence, that the metals undergo a valency change that would then be in agreement with the work of Nicholas & Stevens (68) for Mo^{6+} and Mo^{5+} in nitrate reductase from *Neurospora*. Iida & Yamasaki (69) have determined the molydenum contents of nitrate reductase preparations from *E. coli*.

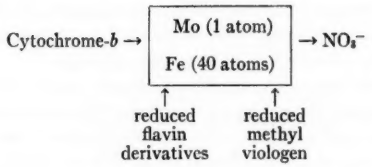

FIG. 2

Sadana & McElroy (70) found that nitrate reductase from *Achromobacter fisheri* used reduced viologen as an electron donor, but the omission of molybdenum from the medium did not affect the reaction. Although they grew the bacteria in peptone medium without added molybdenum, it does not necessarily follow that their cultures were in fact deficient in the micronutrient since the medium was not purified. Thus, further work is required to see whether this organism provides the exception to the thesis presented here that all nitrate reductase enzymes require molybdenum as a terminal link to NO_3^-, the acceptor. They found that iron and bacterial cytochrome functioned in their system as shown in Figure 3.

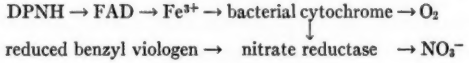

FIG. 3

Fewson & Nicholas (55) found that iron and molybdenum are required for nitrate reduction in *Pseudomonas aeruginosa* when grown anaerobically. A deficiency of either metal reduced enzyme activity and the requirements for both metals increased when the organism was denitrifying. Oxygen had a sparing action on the demand for the two metals. The ratio of the metals was constant during purification of the enzyme. At least half the iron was present as cytochrome-*c* and a requirement was established for it in the electron sequence to nitrate. Evidence was presented for the following (Fig. 4) alternative sequence to the nitrate or oxygen acceptor

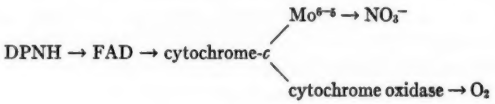

FIG. 4

Using the chromatographic methods of Nicholas & Stevens (68), and by spin resonance measurements, Fewson & Nicholas confirmed that molybdenum probably undergoes a valency change from Mo^{6+} to Mo^{5+}. The inhibition by O_2 of nitrate reductase in cell homogenates is explained by a diversion of electrons via the cytochrome oxidase system.

It is well known that when *Neurospora crassa* assimilates nitrate the enzyme is a flavoprotein containing molybdenum. Recently, Walker & Nicholas (56) showed that submerged felts of *Neurospora* dissimilate nitrate and under these conditions nitrate reductase requires iron as well as molybdenum for its activity. At a later stage of growth when the felts break the surface of the culture solution and emerge into a more aerobic environment there is no longer an iron requirement. The cytochrome-*b* and -*c* contents of the felts were greater in those grown with limited amounts of oxygen. The following scheme (Fig. 5) was suggested by them to explain their results:

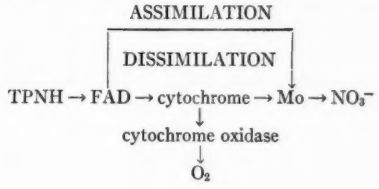

FIG. 5

This scheme is similar to the one proposed for nitrate reduction in *Pseudomonas aeruginosa*. Since O_2 inhibits nitrate reductase in mycelia grown under semi-anaerobic conditions and there is no such effect in mycelia grown aerobically, the above interpretation is supported.

Silver found that nitrate reductase from *Hansenula anomola* was similar to the assimilatory one from *Neurospora crassa* in its dependence on molybdenum for its activity (71). Granick & Gilder demonstrated an obligate requirement for haem compounds for nitrate reduction in *Haemophilus influenzae* (72) as did Lascelles in *Staphylococcus aureus* (73).

Further work has confirmed that molybdenum is a constituent of nitrate reductase in higher plants. Thus Evans & Hall (74) showed that ^{99}Mo accumulated in the purified enzyme and, after dialysis against glutathione and cyanide, the apo-enzyme was activated by sodium molybdate (47 per cent) and to a lesser extent (15 per cent) by iron. Spencer (75) has found a similar molybdenum-dependent enzyme in wheat seedlings. Egami *et al.* (57) have described two types of nitrate reductases in germinating seedlings of *Vignia sesquipedalis* (see Fig. 6).

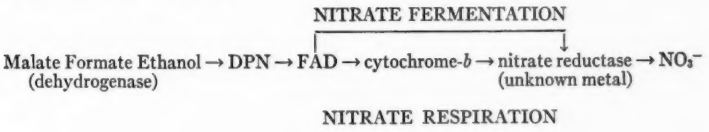
NITRATE FERMENTATION
Malate Formate Ethanol → DPN → FAD → cytochrome-b → nitrate reductase → NO_3^-
(dehydrogenase)
(unknown metal)
NITRATE RESPIRATION

FIG. 6

These workers reported that pre-incubation of the reaction mixture for several hours was necessary to demonstrate enzyme activity and it is there-

fore likely that bacterial contamination may have occurred. The role of cytochrome-*b* is again speculative since in the main it is based on their previous work with *E. coli*. Cheniae & Evans (76) have confirmed their previous results with soyabean plants inoculated with pure cultures of *Rhizobium japonicum* and cultured aseptically in a nitrogen-free medium. An active nitrate reductase is formed in the nodule bacteria and is dependent on molybdenum for its activity. These workers have shown that iron and molybdenum deficiencies reduce the activity of nitrate reductase in the root nodule bacteria of soyabean (77). Whether or not the metals are involved in electron transport as in the nitrate reductase from *Pseudomonas aeruginosa* remains to be determined. Candela *et al.* (78) and Hewitt & Afridi (79) showed that nitrate reductase could be induced in molybdenum-deficient cauliflower and other plants within a few hours after infiltrating the micronutrient into the leaves.

Nitrite.—A nitrite reductase purified over fiftyfold from felts of *Neurospora* contained iron and copper (80). Felts deficient in the two micronutrients had diminished enzyme activity, thus confirming previous work by Medina & Nicholas (81). Nicholas *et al.* (80) suggest that copper might act by coupling the FAD of the enzyme to nitrite since an external supply of Cu^{1+} did reduce nitrite non-enzymatically and this reduction increased in the presence of the enzyme. Thus the Cu^{1+} is regenerated enzymatically. Walker & Nicholas (82) have shown that nitrite reductase from *Pseudomonas aeruginosa* also requires iron and copper for its activity so that it resembles the assimilatory enzyme in *Neurospora* and the results are in agreement with the findings of Chung & Najjar who used the enzyme from *P. stutzeri* (83). It is of interest that the product of the assimilatory and the dissimilatory enzyme is nitric oxide. Horio (84) showed that *P. aeruginosa* grown under anaerobic conditions contains a cytochrome-*c* type pigment (λ 554) and its oxidase. Yamanaka *et al.* (85) suggested that the cytochrome oxidase is oxidised by O_2 or by NO_2^- and that it is inhibited by cyanide. Walker & Nicholas (82) also showed that nitrite reductase from *P. aeriginosa* has cytochrome oxidase activity.

Asano (86) suggested the following scheme (Fig. 7) for nitrite reduction in a halo-tolerant *Micrococcus*. A terminal metal constituent in nitrite reductase, implied by inhibitor studies, was not identified. By analogy with other work this could be copper.

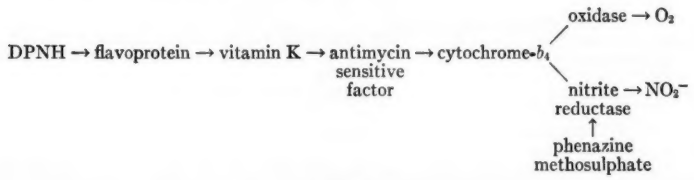

FIG. 7

Roussos & Nason (87) were unable to separate nitrite and hydroxylamine reductases in soyabean leaves although they had evidence that these are distinct enzymes. They claim that both enzymes are inhibited by metal chelating agents and are stimulated by Mn^{++}. They showed that cupric ions at $10^{-3}M$ had little effect on nitrite reductase but when manganese was also added there was a complete inhibition. By contrast, the hydroxylamine reductase was stimulated 50 per cent under identical conditions. Roussos & Nason suggest that the inhibition of the nitrite enzyme might be attributable to the non-enzymatic loss of nitrite from the reaction mixture in the presence of Cu^{++} ions. The metal effects are difficult to interpret since the two enzymes are present together and they appear to base most of their work on the oxidation of reduced nucleotides after adding NO_2^- or NH_2OH. Indeed, it can be argued that they were not in fact measuring nitrite reductase since they state that nitrite was not utilized during the reaction.

Nitric oxide.—Nitric oxide is converted to nitrogen gas in several denitrifying organisms. Chung and Najjar found a nitric oxide reductase in *Pseudomonas stutzeri* and concluded that it was a metalloflavoprotein containing both iron and copper and that it was probably the same enzyme as nitrite reductase (88). Their claim that copper is a functional constituent was based solely on the reactivation of the enzyme after dialysis against salicylaldoxime. It is probable that activation of the treated enzyme was actually a reversal of the inhibition caused by residual salicylaldoxime bound to the enzyme. Since iron and zinc also stimulated the poisoned enzyme, these metals caused a non-specific reversal of the inhibition by the chelate. Fewson & Nicholas (89) showed that a copper deficiency and specific copper inhibitors had no effect on NO reductase in *Pseudomonas aeruginosa* but a deficiency of iron in the cells and inhibitors of the metal depressed the enzyme activity. Iron concentrated in the purified enzyme but copper was absent. The purified enzyme did not utilize DPNH or TPNH (which may explain the low activities in Chung and Najjar's experiments) but reduced pyocyanine was an effective donor. Fewson & Nicholas (90) have observed that nitric oxide, or a compound with which it equilibrates, is readily utilized by bacteria, fungi, algae, and higher plants when grown on nitrate nitrogen but not when ammonia is the sole nitrogen source. Iron deficiency depressed the utilization of NO by these organisms. They also found that N_2-fixing, free-living microorganisms and nodule bacteria, grown without combined nitrogen, also utilize NO.

Hyponitrite and nitrous oxide.—McNall & Atkinson (91) reported that *E. coli* could utilize hyponitrite as the sole nitrogen source and Medina & Nicholas (81) found that hyponitrite was reduced enzymatically in *Neurospora* and the system depended on iron and copper for its activity. This enzyme appeared to be distinct from nitrite reductase since the latter did not utilize hyponitrite; in fact, this substrate inhibited it. Hyponitrite reductase was much reduced in zinc-deficient felts, whereas nitrite reductase was not affected.

Hydroxylamine.—Further work on this enzyme has shown it to be de-

pendent on metals for its activity. Zucker & Nason (92) and Nicholas (2, 3) using *Neurospora* and Spencer, Takahashi & Nason using *Azotobacter* (93) suggest that hydroxylamine reductase is a flavoprotein dependent on manganese for its activity. Roussos (94) and Roussos & Nason (87) claim that manganese stimulates hydroxylamine reductase from soyabean leaves, but the fate of the hydroxylamine is not known. Cresswell & Hewitt (95) observed a rapid enzymic disappearance of hydroxylamine in extracts of vegetable marrow, *Cucurbita pepo*. This they attributed to a peroxidation reaction which was stimulated by manganese, similar to the manganese-dependent peroxidases studied in plants by Kenten & Mann (96). It is clear that the manganese-dependent peroxidation system reported by Cresswell and Hewitt is distinct from the hydroxylamine reducing system. Because the peroxidation of hydroxylamine is very active, it probably masks the hydroxylamine reductase activity. Further purification of the enzyme may well separate the active peroxidase system from the less active hydroxylamine reductase enzyme. Although they suggest that the peroxidase system provides a basis for nitrification of hydroxylamine, from a physiological viewpoint it can be argued that this is unlikely to be important in intact plant cells. This peroxidation of hydroxylamine might well be a feature of disrupted cells only and this point needs further investigating.

Taniguchi, Sato & Egami (63) showed that a purified hydroxylamine reductase from a halotolerant *Micrococcus* was probably dependent on haem-iron for its activity. Walker & Nicholas (97) found that a purified hydroxylamine reductase from *Pseudomonas aeruginosa* required manganese for maximal activity. The purified enzyme first dialysed against sodium diethyldithiocarbamate and then phosphate buffer to remove the chelating agent, was activated by manganese. Although cobalt substituted for manganese, it was only 10 per cent as effective. The manganese requirement was less when either PyH_2, MBH_2, or DPNH was the donor under anaerobic conditions. Although they observed a peroxidation of hydroxylamine in crude extracts under aerobic conditions which was activated by manganese [cf. Cresswell & Hewitt (95)], this phenomenon was not observed either anaerobically or in a purified enzyme and under these conditions hydroxylamine was reduced to ammonia. The function of manganese during hydroxylamine reductase is less clear; it could act as a complexing agent or as a redox catalyst or both. The competitive inhibition by *p*-chloromercuribenzoate of the activation of the purified hydroxylamine reductase by manganese suggests that the metal may be bound to the enzyme by —SH groups.

The following scheme (Fig. 8) presents the possible steps in the inorganic sequence of nitrate reduction and the trace metals so far known to be associated with them.

Ammonia.—Adelstein & Vallee (98) showed that crystalline glutamic dehydrogenase from beef liver contained 2 to 4 gm. atom of zinc per mole protein. Nicholas & Mabey (99) found that the enzyme activity was much reduced in felts of *Neurospora crassa* deficient in zinc, whereas it was unchanged

DISSIMILATION

$$NO_3^- \xrightarrow{Mo,\ Fe} NO_2^- \xrightarrow{Fe,\ Cu} NO \xrightarrow{Fe} N_2$$

ASSIMILATION

$$NO_3^- \xrightarrow{Mo} NO_2^- \xrightarrow{Fe,\ Cu} NO \xrightarrow{?} N_2O_2^- \xrightarrow{Fe,\ Cu} NH_2OH \xrightarrow{Mn} NH_4$$

FIG. 8

in those deficient in iron, copper or manganese. A small reduction of the enzyme (30 per cent) when molybdenum was deficient was attributed to its effect in depressing the nitrate reductase activity, which in turn diminished the production of hydroxylamine (the substrate required to induce the enzyme). The molybdenum effect is abolished when NH_4^+ is substituted for NO_3^- as the sole source of nitrogen.

Amides.—Elliott (100) purified an enzyme from peas that catalyzes two reactions: (*a*) synthesis of glutamine from glutamic acid and ammonia in the presence of ATP, and (*b*) transfer of γ-glutamyl radical to ammonia or hydroxylamine to produce glutamine or glutamo-hydroxamic acid. The enzyme is activated by manganese or magnesium. In reaction (*a*) the activation produced by magnesium increases gradually in a symptotic manner up to 4×10^{-2} *M*. The maximum activation produced by manganese is only a third that by magnesium and reaches a sharp optimum at 5×10^{-3} *M*. High concentrations are markedly inhibitory. In reaction (*b*) 10^{-3} *M* manganese doubles the activity produced by the optimum magnesium concentration 4×10^{-2} *M*. The activation curve for manganese is initially very steep and then falls off whereas that for magnesium is gradual.

Peptides.—Glutathione synthesis in plant mitochondria requires ATP, magnesium and potassium but manganese can substitute for magnesium in most of these reactions. Webster & Varner (101) have studied the metal requirements of two enzymes in wheat germ, the one catalysing the formation of glutamyl cysteine and the other utilizing glutamyl cysteine and either glycine or hydroxylamine to produce glutathione or γ-glutamyl-cysteinyl hydroxamic acid.

Nitrification.—The oxidation of ammonia to nitrite by *Nitrosomonas* and that of nitrite to nitrate in *Nitrobacter* are known to require metals. Engel & Alexander (102) made a notable contribution to the study of the growth requirements of *Nitrosomonas* when they showed conclusively that it would grow quite well in media without suspended $CaCO_3$. All that is necessary is a rigorous control of pH between 7.5 and 8, which they achieved by titrating the acid formed with a sterile solution of K_2CO_3. They showed that the bacterium required iron and this was supplied as a chelate (Chel-138 H·Fe) since an inorganic iron salt would precipitate at alkaline pH. Meiklejohn (103) showed that iron at 4 mg./l. was required for the oxidation of ammonia by *Nitrosomonas*. This apparently large requirement for iron may well have resulted from residual quinoline in the purified media, chelating with the added

iron and thus making it unavailable to the organism. Cell-free extracts, prepared from *Nitrosomonas europeae* by Nicholas & Jones (104), oxidised hydroxylamine to nitrite provided that cytochrome-*c* or some other suitable acceptor was added to the extracts. Cyanide inhibited the oxidation to nitrite when a range of acceptors were used and this combined with recent evidence of a CO inhibition would indicate that both cytochrome-*c* and its oxidase are involved in the final oxidative step to nitrite but not in the dehydrogenation reaction which forms gaseous products. Engel & Alexander (105) found that extracts of *Nitrosomonas* slowly oxidise ammonia and metabolise hydroxylamine rapidly. A metal component was indicated by cyanide inhibition but this was not identified.

In *Nitrobacter*, Lees & Simpson (106) and Lees & Butt (107) reported cytochrome absorption maxima at 589 and at 551 and 520 to 525 mμ on adding nitrite or dithionite; this provides the first evidence for mediation of cytochrome in this reaction. Aleem & Alexander (108) showed that cell-free extracts of *Nitrobacter* prepared by sonic treatment of the cells contained a nitrite oxidising system which was stimulated by iron and inhibited by cyanide. Aleem & Nason (109) confirmed these observations and suggested that nitrite oxidase activity was closely associated with particles. Cytochrome-*c* and its oxidase were implicated in the following electron transfer sequence (Fig. 9).

$$NO_2^- \rightarrow \text{cytochrome-}c \rightarrow \text{cytochrome-}a_1 \rightarrow O_2$$

FIG. 9

Stimulation of nitrite oxidase activity by iron originally reported by Aleem and Alexander was found to be a variable effect. After dialysis of the particulate fraction against cyanide and glutathione, the addition of Fe^{3+} or Fe^{2+} ($4\times10^{-3}M$) specifically activated the enzyme; Cu, Zn, Mn, Co, Ni, W, Mg, V, Mo, and B were without effect. They subsequently showed that antimycin-A, which acts as a powerful inhibitor of the mammalian terminal respiratory chain at a site presumed to be between cytochrome-*b* and -*c*, also caused significant inhibition of the oxidase system. Since the amounts used to obtain this effect were between 100 and 1000 times greater than those required to affect the mammalian succino-oxidase and DPNH oxidase system, more critical evidence is required to establish that it is similar in effect to that on the mammalian system. The fact that 2-*n*-heptyl-4-hydroxyquinoline-N-oxide inhibits the oxidase, cannot in itself be accepted as involving a cytochrome-*b* since Kogut & Lightbown (66) pointed out that its effect on the cytochrome chain is not specific for this or any other cytochrome.

Zavarzin (110) claims that nitrification by *Nitrobacter* is stimulated by molybdenum and slightly inhibited by tungstate when the pH of the culture is 7.7 but at pH 8.8 tungstate stimulates and molybdate inhibits nitrification. At pH 8.1 either micronutrient is stimulatory.

Iron Metabolism

Interest in the correction and causal mechanism of iron chlorosis in plants has been stimulated by the introduction of iron-chelates. This topic is well reviewed by Wallace (111) and by Bould (112) who discuss the use of chelates in culture and field experiments, their stability in aqueous solutions and in soils, and their absorption and translocation in plants. Wallace *et al.* (113) have shown that Chel-138 and Fe-CDTA are more stable in calcareous soils than any other chelates so far tested.

Although early investigators suggested that the metal only was taken up from the chelate, later workers found that the whole molecule was absorbed (7, 111). Wallace *et al.* (114) suggested an equivalent uptake of metal and chelate component by plants but later work from this school suggests a non-equivalent intake. Tiffin & Brown (115) concluded that iron and EDDHA are not absorbed by sunflower plants in equivalent amounts but that iron is released to the roots and most of the chelate component remains in the nutrient solution. Hill-Cottingham (116) made spectrophotometric analyses at intervals on Fe^{3+}-EDTA solution that contained tomato plants and showed a decrease in the concentration of chelated iron without the formation of any free EDTA. He concluded that the entire molecule was absorbed by the roots. Hill-Cottingham & Lloyd-Jones (117) have produced experimental evidence that EDTA is broken down during the utilization of the iron-chelate by tomato plants. Later work by these authors however, showed that the extent to which the iron chelate is absorbed *in toto* or, the iron component only, depends on the nutritional status of the plants (118). Thus, when the plant is deficient in iron more of the metal than the chelate goes in but when the iron content is normal, iron and chelate are absorbed in equimolecular amounts. Doney, Smith & Wiebe (119) found that relatively high concentrations of Ca, Mg, P, Fe, Zn, Cu, and Mn all appear to hinder the translocation of iron applied to the leaves. Brown, Holmes & Tiffin (120) showed that phosphorous, root and chelating agent all appear to compete for the iron supply in the culture solution and thereby exert individual effects in the growth process. They conclude that the primary role of iron chelates in plant nutrition is in making iron available to the root and little evidence that the absorbed chelating agent is an effective activator of Fe within soyabean plants is available. Hill-Cottingham has shown that Fe-EDTA, Fe-HEEDTA and Fe-DTPA all undergo photoreduction and he suggests this provides a mechanism for the release of iron in green leaves (121).

Bicarbonate induces an iron chlorosis in many plant species (122) and this may be a major factor in lime-induced chlorosis. De Kock (123) used several iron chelates with and without bicarbonate and found that at least one chelating agent prevented the bicarbonate chlorosis but did not result in as large a yield as a comparable non-bicarbonate solution. This could be interpreted to mean that bicarbonate may have "sub-clinical" effects on plant metabolism even when there are no visual symptoms. Hale & Wallace (124)

confirmed that four out of eight iron-chelates tested were effective in supplying sufficient iron to soyabean even in the presence of bicarbonate. Bicarbonate, however, reduced the iron content of the soyabean.

Some of the physiological effects of iron chlorosis have been investigated. Thus, De Kock & Morrison (125) showed that the organic acid content of chlorotic plants was often similar whether the chlorosis was produced by genetic effects, by lime, by simple iron deficiency, or even by a virus. Weinstein & Robbins (126) had previously shown that chlorotic plants induced by toxic amounts of manganese, had a high organic acid content similar to that of iron deficient plants. Jackson & Coleman (127) found that an increase in bicarbonate or pH of culture solutions increased organic acid production in bean roots. Malate production accounted for a major proportion of this change produced principally by the phosphoenolpyruvate carboxylase system.

Some biochemical changes have been noted in chlorotic plants. Thus, Miller & Evans (128) studied the effect of bicarbonate on cytochrome oxidase in extracts of several plants. The results show clearly that when compared to other salts, bicarbonate has an inhibitory effect on the oxidase. Plants from cultures containing bicarbonate had markedly reduced cytochrome oxidase activity compared to those given chloride. Their results showed that excessive amounts of bicarbonate salts *in vitro* or *in vivo* inhibited cytochrome oxidase activity. Miller, Brown & Holmes (129) showed that cytochrome oxidase activity of plants was not directly affected by bicarbonate but was much influenced by the phosphate status. Their results seemed to show that bicarbonate increased the soluble phosphorus which in turn inactivated the iron. Another interpretation is that at high phosphorous levels, more CO_2-bicarbonate would accumulate in plant sap and affect the enzyme system. Further work is required to resolve the nature of the inhibition of cytochrome oxidase. Bendall, Ranson & Walker (130) observed that the succinic oxidase system was sensitive to bicarbonate-CO_2 and that the effect was on the oxidation and reduction of cytochrome-*c* in the mitochondria of *Ricinus*. Succinic cytochrome-*c* reductase, DPNH cytochrome-*c* reductase, and cytochrome-*c* oxidase were inhibited markedly by high concentrations of bicarbonate-CO_2. De Kock *et al.* (131) found that peroxidase activity was not markedly different in normal and iron chlorotic leaves whereas catalase was several-fold greater in the green ones. Catalase activity was shown to be depressed when iron was deficient in plants [Abadia (132) and Bannarjee (133)]. Davenport (134) studied the effects of micronutrient deficiencies on haem and chlorophyll levels in normal and chlorotic leaves in a range of plant species.

Rhoads & Wallace (135) suggest that the dark CO_2 fixation by plants producing malic acid and aspartic acid is affected by the bicarbonate-induced chlorosis. The products associated with dark fixation of CO_2 are those reported to increase markedly with bicarbonate or lime-induced iron-chlorosis. Rhoads and Wallace point out that $CaCO_3$ and HCO_3^- would supply an abundance of CO_2. Stolwijk & Thimann (136) noted that barley fixed less

CO_2 in the roots than did two dicotyledons and it is known that the monocotyledons are usually more resistant to lime-induced chlorosis. Bedri *et al.* (137) have also shown that chlorosis-susceptible soyabeans fixed bicarbonate from nutrient solutions at greater rates than did the nonsusceptible ones. Thus there is some evidence that roots fix CO_2 and that among the primary products of this fixation are malic and aspartic acids. Baxter & Belcher (138) also suggested that a decreased decarboxylation as a result of high HCO_3^- levels might cause a metabolic upset resulting in chlorosis. Rhoads, Wallace & Romney (139) showed that malonic acid is a usual constituent of leaves of *Phaseolus vulgaris* and may be increased or decreased under lime-induced chlorosis but Young & Shannon (140) found that with the onset of iron deficiency the acid increased in the leaves. Staples & Weinstein (141) using uredospores and Young & Biale (142) using lemon fruit, found that malonic acid was an early product of the dark fixation of CO_2. Rhoads & Wallace (135) present evidence that the mechanism of increased CO_2 fixation by roots deficient in iron may occur by the phosphoenol pyruvic acid carboxylase system, which combines with CO_2 to produce one mole of inorganic phosphate and one mole of oxaloacetic acid. Bandurski (143) and Jackson & Coleman (144) have shown that this is the main enzyme concerned with CO_2 fixation in soyabean roots although Huffaker *et al.* found that a phosphenol pyruvic acid carboxykinase reaction also occurred, and more recently a CO_2 fixation via γ-ketoglutarate to produce isocitrate has also been demonstrated in the roots (145). For other aspects of the effect of bicarbonate ion on the incidence of iron chlorosis in plants the following papers should be consulted [Bear (122) and Wallace (146)].

Heath & Clark (147) observed that EDTA at 10^{-5} *M* stimulated growth of wheat coleoptile sections. Bennet-Clark (148) showed that EDTA within the range of 10^{-5} to 10^{-3} *M* can act as a growth promoter and suggested the effect might be on the calcium content of the cell wall. Weinstein *et al.* (149) found that EDTA at 10^{-5} *M* stimulated sunflower seedlings but the response to Fe-EDTA, however, was much reduced. Fawcett *et al.* (150), using the free acid and Na salts of EDTA, concluded that growth effects may depend on changes in membrane permeability and cell-wall structure resulting from subacute toxicity, possibly involving metal ions, and that they do not act as true growth promoters. Cleland (151), using ^{45}Ca, has shown no loss of the element from the cell walls of *Avena* coleoptile and those of *Zea* maize treated with auxin. Burström (152) suggested that gibberellic acid reverses iron-induced inhibition of cell elongation in the light but has no effect in the dark.

Iron deficiency reduced catalase, peroxidase, cytochrome-*c* reductase, and oxidase (in that order), DPNase activity, and DPNH and TPNH diaphorases in *Neurospora crassa* (153). Bacon *et al.* have shown that aconitase is reduced in plants deficient in iron (154).

Weinstein & Robbins (126) reported that catalase and cytochrome oxidase of sunflower leaves were low when either iron was deficient or manganese

was present in toxic amounts. Healy, Cheng & McElroy (155) state that toxic amounts of cobalt in *Neurospora* cultures result in enzyme patterns in the felts similar to those obtained with iron deficiency.

Haem compounds in plants have been further investigated. According to Hartree (156), haematin is present in all plant cells except for strict anaerobes, and the total haematin content of green leaves can be ten times that of non-chlorophyll containing plants. Cytochrome-*c* and its oxidase are widespread in plants [James & Boulter (157)]. Martin & Morton (158) identified cytochrome-b_3 in the microsomes of silver beet, onion, and *Arum spadix*. They suggest it is similar to the cytochrome-b_3 isolated from leaves by Hill & Scarisbrick (159). It is reduced anaerobically by DPNH and TPNH and rapidly reoxidised in air. Cytochrome-b_3 may correspond to the cyanide-insensitive respiratory system of *Arum spadix*, although James (160, 161) attributes this to a flavoprotein. Cytochrome-*f*, first discovered by Hill and Davenport, has now been found in a range of green plants including algae. Lundegårdh (162) found that cytochrome-*f* was oxidised in the light and reduced in the dark and that ascorbic acid also reduced it. However, cytochrome-*b* and -*c* in the leaves were not as markedly affected by light. Arnon, Whatley & Allen (163) suggested that cytochrome-*f* may be involved in photosynthetic phosphorylation under anaerobic conditions in the presence of ascorbic acid.

Haskins *et al.* (164) and Tissières & Mitchell (165) studied the cytochrome contents of various strains of *Neurospora crassa*. The wild type contained cytochromes-*b*, -*c*, and $a+a_3$ whereas a "poky" strain was deficient in *b* and $a+a_3$ but contained large amounts of *c*. The "poky" types grew slowly and had a cyanide-insensitive respiration. Other strains were deficient in either $a+a_3$ or *b*, and in some *c* was also reduced. The "poky" type had a high succinic oxidase concentration during early growth which reverted to normal levels after six days growth, a low oxidase and high succinic dehydrogenase. An enzyme that destroyed cytochrome-*c* was active during early growth in the "poky" strain. Haemoglobin, first found in nodules of leguminous plants [Kubo (49), Keilin & Wang (166), Virtanen (167)], has now been identified in nodules of nonlegumes by Davenport (168). Keilin & Hartree (169) have shown that catalase, peroxidase, and metmyoglobin oxidise substrates in the presence of H_2O_2. Catalase appears to favour oxidation of alcohol by peroxide and the enzyme seems to protect ascorbic acid from a peroxidation catalysed by peroxidase in the absence of free metal ions. It is of interest that both enzymes catalyze oxidation of nitrite. Peroxidation mechanisms involving manganese in plant tissues have been thoroughly investigated by Kenten & Mann (170).

Phosphorus Metabolism

Spencer (171) suggested that molybdate was an inhibitor of acid phosphatase activity in tomato plants *in vivo* and *in vitro* and this he believes accounts for the apparent molybdenum requirement of plants grown with

NH_4^+ since under these conditions there is no longer a molybdenum requirement for nitrate assimilation. Hewitt (8, 9) has contested this view, since normal plants contain about 10^{-7} *M* molybdenum and at this level the effect on acid phosphatase in tomato is negligible. No effect of molybdate on acid phosphatase was detected in leaves infiltrated with molybdate at 10^{-4} *M* and assayed 24 hr. later (8). Nicholas & Mabey (172) suggest an alternative reason for a small molybdenum requirement in *Neurospora* grown on NH_4^+. They showed that catalase and peroxidase activities were much reduced in the molybdenum-deficient felts, since their common enzyme substrate H_2O_2 was depressed. This was linked to a reduced flavoprotein activity since the total FAD was reduced and the nitrate reducing flavoprotein enzymes were not developed. Nicholas & Commissiong (173) found that excess molybdenum depressed acid phosphatase activity. Molybdate inhibited acid phosphatase competitively *in vitro* presumably by forming a molybdo-phosphate complex with the substrate; copper dispersed this complex and restored enzyme activity to normal values. Hewitt & Tatham (174) claim that a deficiency of zinc with nitrite or ammonia as the nitrogen source, and of copper with nitrite only, increased acid phosphatase activity of leaf extracts of a range of higher plants. A deficiency of molybdenum resulted in a twofold increase in enzyme activity when plants were grown with nitrate. They conclude that accentuated enzyme activity resulted from increases in total amounts of phosphatase produced during growth and that different combinations of deficiencies and nitrogen supply produced different specific responses in terms of enzyme synthesis. Their experimental data, however, do not differentiate between metal effects in total enzyme production or, alternatively, on the preformed enzyme.

Possingham (175) found a marked decrease in the ratio of organic to inorganic phosphorus in plants deficient in molybdenum over a 4-day period although the concentration of total phosphorus was unchanged. The total organic phosphorus per plant increased after two days when molybdenum was given to plants deficient in the element. Hewitt (176) found that the proportion of organic to inorganic phosphorus decreased in molybdenum-deficient cauliflower grown with nitrite, ammonia, urea, or glutamic acid. Acid-labile phosphorus and the alcohol-insoluble barium fraction were decreased in proportion to the total by a molybdenum deficiency, whereas there was a marked increase in free and pyrophosphate-combined 5′-adenylic acid. He claims that changes in the ratio of organic to inorganic phosphate could occur either with increased phosphatase or impaired phosphorylation but no experiments were reported to explore these and other possibilities.

Kinsky & McElroy (177) and Nicholas & Scawin (178) found that purified nitrate reductase from *Neurospora* was stimulated about 50 per cent by phosphate. Orthophosphate could be replaced by arsenate, tellurate, or selenate and to a lesser degree by sulphate or silicate. These authors suggested that these ions might bind the molybdate to the apo-enzyme and facilitate electron transfer. The finding that the requirement is at the point

where molybdenum acts in the system would support this view. Kinsky & McElroy (177) and Hewitt (8, 9) have suggested that the oxidation of Mo^{6+} is considerably influenced by these anions and that they would facilitate the reduction of the micronutrient by lowering the oxidation-reduction potential. This was shown in the author's laboratory since phosphomolybdate^{5+} acts as an effective electron donor for nitrate reduction using a very purified enzyme having a phosphate requirement. It is of interest that phosphate is essential for all the flavoprotein enzymes in the inorganic route for reduction of nitrate to ammonia [Medina & Nicholas (81)].

Other Metabolic Effects

Manganese—Anderson & Evans (179) found that manganese-dependent isocitric and malic enzymes in *Phaseolus vulgaris* were present but inactive in extracts prepared from manganese-deficient plants and were immediately reactivated on adding manganese to the crude extracts. This result contrasts with the absence of nitrate reductase when molybdenum is deficient. Phosphoenol pyruvate carboxykinase found in plants by Mazelis & Vennesland (180) is activated by manganese or magnesium. Dialysed extracts of the enzyme from turnip, cauliflower, squash, or lupin tissues were activated by 10^{-2} *M* manganese or magnesium. Mazelis (181) concluded that adenylic kinase from mitochondria bound metals more firmly than did a similar enzyme from spinach chloroplasts and it was correspondingly less sensitive to inhibition by fluoride. Humphries (182) found that glutathione reductase from wheat germ required manganese at 3×10^{-3} *M* for its activity. Kenten (183) showed that in extracts of waxpod bean roots a mole of indoleacetic acid was oxidised by a mole of O_2 when peroxidase and *p*-cresol were present. A manganese salt between 10^{-6} to 3×10^{-5} *M* stimulated the reaction. Waygood, Oaks, & Maclachlan (184) suggested that 10^{-9} *M* Mn^{++} activates the oxidation of indoleacetic acid in phosphate extracts of wheat leaves. A monophenol is also required for the complete oxidation of the substrate. They suggest an oxidative decarboxylation of the inodoleacetic acid catalysed by free trivalent Mn^{+++}, and the reoxidation of divalent Mn^{++} by an oxidised product of the monophenol produced in the presence of peroxidase and H_2O_2. The H_2O_2 may be formed during aerobic dehydrogenation of the decarboxylated indole derivative. The contradictory reports in regard to manganese stimulation of the enzyme is probably due to the type of buffers used since it is well known that citrate and pyrophosphate chelate with manganese and this affects its oxidation-reduction potential. Galston & Dalberg (185) have considered in detail the role of the flavoprotein oxidase enzyme and manganese in the oxidative inactivation of indoleacetic acid in leaves. Waygood & Maclachlan (186), however, suggest that peroxidation of Mn^{++} is brought about by an organic peroxide derivative of the decarboxylated indoleacetic acid formed during oxidation. The physiological importance of manganese in the nutrition of agricultural plants is described by Vlasyuk (187). Romney & Toth (188) discuss the translocation of ^{54}Mn

from the leaves to the meristematic tissues and Boken (189) has followed the movement of manganese from the tops of cereals to the roots.

Eyster, Brown & Tanner (190) showed that *Chlorella* required 10^{-7} p.p.m. manganese for heterotrophic growth and 10^{-4} *M* for autotrophic growth. Autotrophic growth, Hill reaction, and photosynthesis responded equally when increments of manganese were added to manganese-deficient cultures. Similar results were obtained with *Scendesmus quadricauda*, *Nostoc muscorum*, *Porphyridium cruentum*, and *Lemna minor*. In tests with the green alga, *Ankistrodesmus braunii*, Kessler *et al.* (191) found that manganese deficiency markedly inhibited photosynthesis and delayed light emission while fluorescence increased. They suggest that manganese is important in the O_2-evolving system (192). Bertrand & Wolf (193) claim that manganese is important for the utilization of citric acid in *Aspergillus niger* while Bromfield (194) has noted the relation between manganese deficiency in *Corynbacterium* and the production of H_2O_2.

Saz & Slie (195), Rosen (196), and Gray (197) observed that manganese was able to reverse the inhibitory effects of aureomycin on bacterial nitroreductase or that of streptomycin on the growth of plant tissues. Saz & Martinez (198) suggest that the relative resistance of strains of *E. coli* to aureomycin is closely related to their flavin and manganese requirements and that aureomycin competes with flavins for the metal.

Zinc.—Bertrand & De Wolf (199) showed that *Aspergillus niger* required zinc for the synthesis of tryptophan and claimed that cadmium could substitute for it in the enzyme. They also showed the importance of the element for glucose-6-phosphate and 6-phosphogluconic dehydrogenases in the fungus (200). Schneider & Disselkamp (201) used ^{65}Zn to follow distribution of the micronutrient in various fractions of the same fungus. Medina & Nicholas (202) found that hexokinase from *Neurospora crassa* required zinc for its activity and a deficiency of the element reduced DPNH and TPNH diaphorase and increased acid phosphatase in the felts. Kalyanasundaran & Saraswathi-Devi (203) concluded that the addition of zinc beyond that required for optimum growth increased the production of an unidentified antibiotic substance by *Fusarium vasinfectum*. The stimulation of cytochrome pigments by zinc in *Ustilago sphaerogena* occurs at concentrations in excess of normal growth requirements (204).

Vallee and his associates (205, 206) have made a thorough investigation of zinc-containing metallo-enzymes, including alcohol dehydrogenase of yeast (YADH) and liver, glutamic dehydrogenase from beef liver, and lactic dehydrogenases of rabbit skeletal muscle. Mathries (207) showed that alkaline phosphatase from kidney contains 0.15 per cent zinc which is firmly bound to the protein. It is likely that these enzymes, which are also present in various plant tissues, also contain the metal. Thus Nicholas & Mabey (172) reported that glutamic dehydrogenase activity was depressed in zinc-deficient felts of *Neurospora* and the enzyme was restored to normal values by adding the metal *in vivo* to felts deficient in the element. Vallee (206)

showed that crystalline YADH from baker's yeast contains 4 atoms of zinc per molecule and he considers the enzymic reaction to be as shown in Figure 10.

$$\text{YADH}\cdot\text{Zn} + \text{DPN} \rightleftarrows \text{YADH}\cdot\text{Zn}\cdot\text{DPN}$$
$$\text{YADH}\cdot\text{Zn}\cdot\text{DPN} + \text{C}_2\text{H}_6\text{OH} \rightleftarrows \text{YADH}\cdot\text{Zn}\cdot\text{DPNH} + \text{CH}_3\text{CHO} + \text{H}^+$$
$$\text{YADH}\cdot\text{Zn}\cdot\text{DPNH} \rightleftarrows \text{YADH}\cdot\text{Zn} + \text{DPNH}$$

FIG. 10

Zinc is thought to bind DPN or DPNH to form the active enzyme-coenzyme complex. Data with inhibitors indicate that the rates of dissociation of the coenzyme from the zinc sites are the rate-limiting steps in the overall reaction. The metal appears to be required both for the catalytic properties and in the stabilization of the enzyme protein. Kägi & Vallee (208) have recently shown that YADH (molecular weight of 151,000) can be resolved into four subunits. The empirical structure of yeast ADH is $(\text{YADH})_4\text{Zn}_4(\text{DPN})_4$, where YADH represents a single unit with a molecular weight of 36,000. The zinc atoms are thought to stabilize the quaternary structure of the enzyme. Van Eys & Kaplan (209) and Van Eys, Ciotti & Kaplan (210) demonstrated the binding of DPN^+ and DPNH by yeast alcohol dehydrogenase and proposed a mechanism involving zinc-pyrophosphate and other bonds. Enolase, which catalyzes the dehydration of *d*-2-phosphoglyceric acid to phosphoenol pyruvate, is activated by magnesium, zinc, and manganese. Malmstrom (211) studied the binding of zinc to enolase and found that one atom of zinc or manganese may combine per mole of enzyme protein to give an active enzyme. In the presence of 1×10^{-4} *M* zinc, four moles of substrate are bound to the enzyme but three moles are probably bound non-specifically. Vallee (206) has suggested that zinc may have purely structural implications in α-amylase from *Bacillus subtilis;* the enzymic activity of the zinc-free monomer is identical with that of the zinc-dimer.

Copper.—The possible role of copper-activated oxidase systems as terminal electron transport systems in plants has been debated on numerous occasions. James (212) concluded that cytochrome oxidase is the terminal enzyme in barley embryo but during early development in young roots (ten days after germination) it decreased and an active ascorbic acid oxidase developed. Polyphenol oxidase was not detected in these tissues. James & Boulter (213) observed a transition between the presence of cytochromes-*a*, -*b*, and -*c* and cytochrome oxidase and of ascorbic acid in barley roots between three and six days after germination. The cytochrome system was not detected spectroscopically in the roots after six days. Mapson, Isherwood & Chen (214) found that mitochondria from pea seeds do not contain ascorbic oxidase but it appears within 120 hr. of soaking the seed. Thimann, Yocum & Hackett (215) demonstrated a change in potato tubers from a cytochrome oxidase to a flavoprotein oxidase system. Mapson (216) observed that plants

lacking polyphenol oxidase usually have an active ascorbic oxidase. Nason, Wosilait & Terrell (217) claim that DPNH or TPNH are oxidised in the presence of an enzyme from pea seeds by a labile oxidation product of ascorbic acid produced by the action of ascorbic acid oxidase but distinct from dehydroascorbic acid. This might indicate a possible link between the reduced pyridine nucleotides and a copper-dependent terminal oxidase. There is insufficient critical evidence at the present time to assess the importance of the copper-oxidase systems as alternative terminal acceptors in plant respiration.

Green, Basford & Mackler (218) found a high constant ratio of copper to haem iron in the electron transport particle prepared from beef heart mitochondria. Sands & Beinert (219), using paramagnetic resonance (E.P.R.) spectroscopy, have identified a signal at $g=2.05$ in cytochrome oxidase preparations of mitochondrial particles from animal tissues. This signal, which decreased in intensity in the presence of substrates of the oxidase, is characteristic of Cu^{2+}. This provides the first evidence for a possible valency change in copper during cytochrome oxidase action. By analogy with the animal system, studies with cytochrome oxidase from plant tissues may show that it also contains copper. Bendall & Hill (220), James & Beevers (221), and James & Elliott (222), who studied respiration in *Arum spadix*, reported on an autoxidizable cytochrome-b_7, and a flavoprotein system, both cyanide insensitive. They also found small amounts of a cyanide sensitive cytochrome-*c* and a cytochrome oxidase system. It is of interest that James & Leech (223) found that isolated chloroplasts of *Caulerpa prolifera* and *Vicia faba* contained cytochrome-b_6 and *-f* only and no other cytochromes.

Boron.—The effect of this micronutrient on carbohydrate metabolism was reviewed by Gauch & Dugger (224). They suggest that boron facilitates the movement of sugars in plants by forming a permeable boron-sugar complex or by joining the cell membrane in a way that makes it more permeable to sugars. Dugger, Humphreys & Calhoun (225) found that boron affected the sugar-starch ratio of leaf discs of bean and that phosphorylase was inhibited by the micronutrient. Spurr (226) examined the petioles of boron-deficient celery prior to phloem necrosis and found the walls of phloem parenchyma thicker and the collenchyma thinner than normal. Odhnoff (227) and McIlrath & Skok (228) propose that boron influences cell development. The former thinks that boron controls polysaccharide formation but does not believe it affects sugar translocation. McIlrath & Palser (229) also conclude that the effect of boron on translocation is secondary, being caused by necrosis of the phloem in deficient plants. Whittington (230) suggests that boron controls the rate of cell division in germinating beans and he also reinforces Winfield's hypothesis that boron is required in pectin synthesis (231) although there are many objections to this view (226, 227, 232). Scott (233) suggests that boron competitively inhibits the formation of starch by combining with the active site of phosphorylase. He suggests a protective function for the minor element in that it prevents the excessive polymerisa-

tion of sugars at sites of sugar synthesis. He thinks it significant, therefore, that the highest concentration of boron is in the leaves.

Pejve (234) investigated the effect of boron on ascorbic and polyphenol oxidases and peroxidase in tomato, oat, and wheat seeds. Skok (235) showed that withholding the nutrient for short intervals prior to x-irradiation with doses that normally produce radiation symptoms alleviated radiation injury in sunflower plants. Myszka (236) and Novoderzhkina (237) showed that boron treatment of *Brassica* crops and potato respectively increased vitamin C content, whereas other workers noted a decrease in the vitamin content of plants given large amounts of the micronutrient. Since ascorbic acid is known to vary markedly with small changes in the environmental conditions, no reliance can be put on reports of fluctuations in this vitamin resulting from micronutrient deficiencies unless the plants have been grown under rigidly defined conditions in growth chambers. Daniel & Varoczy (238) have noted beneficial effects of boron on germination of pollen grains of 56 species of fruit, vegetables, and ornamental and wild plants. Gorter (239) showed that boron increases root production in *Phaseolus* cuttings in association with indole and indole-3-acetic acid. Azimov (240) determined the effect of boron treatment on the synthesis/hydrolysis ratio of sucrose in leaf discs of peanut plants.

Epilogue

It is beyond the scope of this review to anticipate future developments in studies with minor elements in plants. Certain trends, however, are apparent. Thus, the agronomic importance of trace metals will be further emphasised as underdeveloped areas of the world are brought into a more "artificial" type of crop cultivation. Webb (241) has already stressed the importance of minor elements in tropical and subtropical agriculture, since their application often makes a difference between a good crop yield and no crop at all.

On the academic side, the search for new minor element requirements is likely to continue. More sensitive members of the plant kingdom may be found that require as yet unidentified elements for their metabolism. In this connection a study of the non-economic crops, especially those growing in unusual habitats and the marine microflora, should be rewarding. The physical difficulties in eliminating ultra-micronutrients from the environment of higher plants, to prove or disprove their essentiality, may become insuperable despite the use of costly growth chambers, automatic irrigation devices based on a plastic regime, and with such aids as radioactivation analyses to check the purity of materials used.

The fashionable approach nowadays is to establish a requirement for minor elements in plant metabolism. This method also has its difficulties and limitations. It is important both to pinpoint the site of action of minor elements in a vital metabolic process and to understand the role they play in association with the enzyme protein and other cofactors when present.

The metal should also function in an indispensable metabolic system and not in one that is of no importance *in vivo*. A metal requirement for an enzyme cannot be shown conclusively from one line of evidence only, so several types of experiments need to be done to establish a metallo-enzyme. These include (*a*) deficiency studies—useful only in eliminating metals that do not affect the enzyme, (*b*) concentration of metals in the purified enzyme to the exclusion of others, (*c*) reversible dissociation of metals from the enzyme, together with a reactivation of the apo-enzyme by returning the appropriate element or elements, (*d*) activation of the purified enzyme by metals—although this effect could result in the removal of an inhibitor, and (*e*) for transition metals, use of electron-spin resonance measurements to determine valency changes during enzyme action—although matching signals so obtained with those of inorganic models is not clear-cut. Thus, unequivocal evidence that minor elements are functional constituents of enzymes is difficult to achieve. The presence of metals in very pure enzyme samples does not necessarily imply a function for them during enzyme action. For example, copper found in butyryl CoA dehydrogenase was at first thought to be essential for its action but further purification removed the element without affecting enzyme activity. Singer & Massey (242) have pointed out that a concentration of metals in an enzyme could be derived from reagents used in its purification since proteins act as nonspecific chelating agents. This is not altogether true since in the reviewer's experience it is far easier to lose metals during fractionation than to gain them. Gels used during purification readily adsorb metals and compete effectively for them with some proteins. Incorporation of labelled micronutrients in the growth medium, followed later by the determination of the tracer in purified enzyme fractions of the organism, invalidates the criticism that the minor elements in enzymes could be derived from materials used during fractionation. In the reviewer's experience, extraneous metals seldom accumulate in purified enzyme fractions. This is routinely checked in this laboratory by incorporating a variety of labelled trace metals in the culture solutions in which the organisms are grown and then determining whether they accumulate in the particular enzyme. In the author's opinion, the methods suggested above, when used together, can provide fairly conclusive evidence that the metal is required for enzyme action but the precise mechanism involved must await further knowledge of the protein structure, the main component of any enzyme.

Thus far investigations on the role of minor elements in plant metabolism have been concerned mainly with isolated enzyme systems. The micronutrients have a profound effect on membrane structures (including permeability barriers within cells, cell organization, etc.) that are not amenable to investigation by present day biochemical or biophysical methods. Advances in this area will depend on the development of suitable techniques to study cells and their contents *in vivo*.

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SOIL-PLANT RELATIONSHIPS IN ION UPTAKE[1]

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INTRODUCTION

Soil-plant relationships in ion uptake concern the factors that determine how a given element in the soil finds its way into the metabolic system of the plant. These relationships may differ for each element. However, there are certain generalizations common for all elements in the description of soil-plant relationships. Chemical and physical processes are similar but magnitudes vary.

The purpose of this review is to point out the chemical and physical processes involved in the transport of an ion from its place of origin in the soil to its place of utilization in the plant. The discussion will include the relationship between processes as well as the processes themselves. The organic fraction of the soil which may contribute substantial amounts of nitrogen and lesser amounts of other elements such as S and P will not be considered.

DYNAMICS OF THE SOIL-PLANT SYSTEM

Plant growth requires a continuous net shift of ions from the soil system into the plant; i.e., there is a steady input of ions from the solid phase into the soil solution, and a continuous metabolic removal of ions from the soil solution by the plant. For any short time interval in a given environment, the net release of ions by the soil equals the net amount assimilated by the plant. At any time t the soil-plant system is in a steady state with regard to each of the elements but changing the supply of the element by fertilization or plant removal alters this steady state level. In this steady state system, one or more steps may be slower than the others and limit the rate of the overall process.

Ion uptake by plants from a soil system may be divided into four steps: first, the release of the ion from the solid phase into the soil solution; second, the movement of the ion from any point in the soil solution to the vicinity of the root; third, the movement of the ion from the vicinity of the root into the root; and fourth, the movement of the ion to the top of the plant. This sequence is described in Equation 1, where M is the ion studied.

$$\mathrm{M(soil)} \rightleftharpoons \mathrm{M(solution)} \rightleftharpoons \mathrm{M}\left(\begin{array}{c}\text{vicinity}\\ \text{of root}\end{array}\right) \rightleftharpoons \mathrm{M(absorbed)} \rightarrow \mathrm{M(plant\ top)} \qquad 1.$$

[1] The survey of literature pertaining to this review was concluded in September 1960.

Each of the transfer steps involves both rate constants and concentrations. Furthermore, each step takes place at a rate that is controlled by measurable factors of either soil or plant. One or more of the transfer steps may be rate limiting to the overall process.

THE SOIL SYSTEM

Solid Phase

The primary source of the nutrient ion M is the solid phase. The relationship of the ion between solid phase and soil solution may be described or inferred either by a mineralogical and chemical identification or by the kinetics of solid-phase—soil-solution relationships.

Mineralogical and chemical identification.—Mineralogical and chemical identification is one approach to characterizing M(soil). The most abundant geochemical form of the nutrient elements is not necessarily the most abundant form found in soils. Thus, the principle mineralogical form of molybdenum is molybdenite, MoS_2 (38). The secondary molybdates are readily formed by weathering (38), however, and molybdenum in soils is retained in the molybdate form by clay minerals (39, 109) or by inclusion in or sorption on hydrous oxides (60, 108, 109). The oxides of manganese are the most important mineral forms (38), but in soils manganese is found as either the exchangeable ion, reducible manganese, or in the form of the higher oxides (61, 63, 86, 166). Phosphorus occurs most abundantly as apatites (38), and small amounts of these minerals can generally be found in calcareous soils (149). Calcareous soils also contain other calcium phosphates, however, and phosphorus occurs as iron and aluminum phosphates in acid soils (43, 119, 149). The principle mineral forms of calcium (carbonates, phosphates, silicates, sulfates, and fluoride) and of magnesium (carbonates and silicates) (38, 168) probably also occur in calcareous soils. However, in both acid and calcareous soils, calcium occurs as the exchangeable ion. Potassium is present in soils in the primary minerals, feldspars, and micas as well as in association with the clay minerals such as illites, montmorillonites, vermiculites, and kaolinite (2, 3, 37, 45, 131, 132, 142, 168, 175, 187). Although mineral nitrate deposits occur, soil nitrogen is primarily in the organic form. Inorganic soil nitrogen may occur as the exchangeable ammonium ion and appreciable amounts may be associated with micaceous clays (81, 140, 160, 177, 178); the inorganic chemistry of nitrogen is also important in delineating its plant availability (6, 76, 126, 177, 183).

The solubility in various extracting agents is useful in identifying the mineralogical and chemical components. Potassium, for example, has been separated into water soluble, exchangeable, fixed, and lattice forms (101, 187). A similar type of separation has been made for molybdenum (1, 39). The difference in zinc solubility, noted in an alkaline soil with change in pH, was used to postulate the formation in soils of soluble zincates of sodium and potassium and an insoluble zincate of calcium (110). At constant CO_2 pressure the calcareous material in soils is more soluble than pure calcite (151).

Chemical extraction methods also are used to estimate the relative amounts of the several phosphorus mineral forms in soils (12, 29, 42, 75).

Solubility products are specific for given chemical compounds and can be used to identify the solid phase. The solubility products of variscite (31, 128), and strengite (28), and the possible occurrence of iron and aluminum phosphates (32, 115, 189, 190) in soil systems have been determined. In highly fertilized calcareous soils metastable octa-calcium phosphate may be formed, as may dicalcium phosphate, in the vicinity of the fertilizer particle (5, 33). The solubility of phosphorus in calcareous soils was expressed recently in terms of the mean activity of dicalcium phosphate (34).

Solid phase-soil solution relationships.—Another way to characterize M(soil) is to measure M(soil) and M(solution) and quantitatively describe their interrelationships.

(*a*) *Intensity vs. capacity.*—The instantaneous supply of nutrient to the root consists of the concentration in the soil solution that is in contact with the root (4, 44, 82, 123, 148, 180, 186). This is an intensity factor analagous to hydrogen ion concentration in the hydrogen system.

Reported composition of the soil solution for various soils is summarized in Table I. Of the major nutrient ions (Ca, Mg, K, N, P, and S) the cations are usually present in concentrations approximating 10^{-3} to 10^{-4} *M*. Phosphorus usually is in the lowest concentration, commonly about 10^{-5} to 10^{-6} *M*. Thus, the soil solution phosphorus in most agricultural soils must be renewed many times per day by the solid phase M (soil) in order to give the total uptake of phosphorus noted in plants (67, 154). Less rapid but continued renewal is also required for the cations.

The capacity of M(soil) to renew the ions in solution is a capacity factor analagous to the total acid in the hydrogen system. The magnitudes of the

TABLE I

SOIL SOLUTION COMPOSITION

Element	Range of All Soils*	An Acid Soil (180)	A Calcareous Soil (158)
		(moles/liter$\times 10^3$)	
Ca	0.5–38	3.4	14
Mg	0.7–100	1.9	7
K	0.2–10	0.7	1
Na	0.4–150	1.0	29
N	0.16–55	12.1	13
P	<0.001–1	0.007	<0.03
S	<0.1–150	0.5	24
Cl	0.2–230	1.1	20

* (23 to 26; 50, 90, 139, 156, 157, 158, 180.)

intensity and capacity factors and the relationship between them, M(soil) $\rightleftharpoons$M(solution), distinguish the nutrient ion system in one soil from the nutrient ion system in another.

This transfer of ions between phases is apparently not rate limiting for either phosphorus or cations in the overall ion uptake process described in Equation 1. Thus, for two acid soils and one calcareous soil, the rate of formation of soil solution phosphorus was greater by a factor of at least 250 than the rate of absorption of phosphorus by the plant from the soil solution (68). Exchange data with ^{32}P gave similar results (66, 133) and the rate of exchange of cations between solid and solution phases is also rapid (74, 87). Borland & Reitemeier (15) found equilibrium of calcium within one-half hour, the shortest test period used, while Barbier *et al.* (9) concluded that for calcium and possibly the other exchangeable cations as well, the ions in solution and on the solid phase exchange a number of times within several minutes.

The significance of the exchange rates mentioned above is that the rate of release from the solid phase is not rate limiting in Equation 1. Even under conditions of active nutrient uptake by the plants, M(soil) is essentially in equilibrium with M(solution) at the soil—soil-solution interface. Although the transfer between phases is not rate limiting, this does not imply that the ion concentration in the soil solution is sufficient for maximum growth. The similarity in effectiveness of various forms of zinc added to the soil (14, 171, 174) reflects this relationship (14). Furthermore, equilibrium studies in the presence and in the absence of the solid phase gave similar growth and/or uptake of cations and anions (44, 123, 148, 180). Thus, the composition of the equilibrium solution M(solution) characterizes the instantaneous plant root environment.

(*b*) *Surface reactions.*—Although ion uptake by the plant root reflects the concentration in solution, the ion supplying power of a soil is a function not only of this concentration but also of the capacity of the solid phase M(soil) to replace these ions in the soil solution as they are removed by the growing plant. Many soil studies have dealt with the rate of release of ions from a solid phase (52, 68, 91, 134, 141, 143, 144, 169). The Ca, Mg, Na, K (175, 176), and B (77) supplying power of various primary minerals, for example, was related to the amount released to ion exchangers. On the other hand, a continuous percolation technique showed that the rate of release of nonexchangeable potassium by 41 soils was approximately constant and ranged from 0.3 to 2.7 lbs. K_2O/acre/hour (134). This steady-state rate of release was a characteristic of each soil since it did not change with the amount of fertilizer added or the manner of drying the soil. The rate of release was about 1/30 that found for soil phosphorus (66, 68, 134).

The rate of release of ions from the solid to the solution phase may be more precisely defined by means of reaction kinetics. Thus, potassium release from biotite has been described in terms of an initial zero order reaction followed by a first order reaction (52, 141) that may be diffusion controlled (143). The results showed a definite relationship between the slope of the

rate-of-release curve and the degree of weathering of the material used (52). Similarly, the displacement of phosphorus into the soil solution (169) and the reaction of cobalt with montmorillonite (91) follow first order reaction kinetics.

The M(soil)-M(solution) relationship also has been described in terms of equilibrium sorption reactions (40, 120). A Freundlich isotherm described the desorption of adsorbed ammonia from soils (30) and the adsorption of copper by clay minerals (85). The Langmuir isotherm described the reaction in soils of P (33, 67, 150), S (112), B (13, 82, 83), Na (17), and Cu (182), and the BET isotherm described the absorption of ammonia by clays and mucks (140). Calculated values of the surface phosphorus based on the Langmuir equation and the surface phosphorus determined by equilibration with ^{32}P for 48 hr. were in good agreement for four low-phosphorus-fixing soils (67). In the low range of solution phosphorus concentrations, phosphorus adsorbed on several acid and calcareous soils followed a Langmuir isotherm, was readily exchangeable with ^{32}P, and occurred as a monolayer (33, 150). Similarly, the removal of Na (17) or B (82) from soils and the amount of a trace element that may be added without injury (82) may be predictable.

Recently, using mass action considerations, a similar relationship between the capacity factor M(soil) and the intensity factor M(solution) was developed for phosphorus (169), calcium or strontium (84), and sodium (65) as shown in Equation 2:

$$\text{M(soil)} = -K_m \frac{\text{M(soil)}}{\text{M(solution)}} + \Sigma\text{M(soil)} \qquad 2.$$

where K_m = an apparent dissociation constant for the particular system specified; ΣM(soil) = the amount of M adsorbed per gram of soil when all the adsorption sites are saturated with M; M(solution) = concentration of M in solution; and M(soil) = amount of M adsorbed per gram of soil.

A plot of

$$\text{M(soil) vs.} \frac{\text{M(soil)}}{\text{M(solution)}}$$

should give a straight line with an intercept of ΣM(soil) and a slope equal to K_m. Edsall & Wyman (51) discuss this treatment for equations of binding describing "reversible equilibria." They are formally analogous to steady-state equations used in enzyme kinetics and ion uptake (49, 70, 71, 72, 78, 92).

A plot of phosphorus adsorption data according to Equation 2 is shown in Figure 1. Graphical separation (92) of the curve into its components showed at least two forms of adsorbed soil phosphorus. Since the slope of the lines is an apparent dissociation constant, the form of phosphorus that predominates at low solution concentrations is held 100 to 1000 times as tightly as the form that predominates at high phosphorus concentrations. The constants in the relationship between M(soil) and M(solution) as given

in Equation 2 characterize the soil phosphorus system since the constants are independent of the soil/solution ratio in the range of 1:5 to 1:2000, and of the weight of soil (169). This equilibrium characterization of the soil phosphorus agrees with the nonequilibrium kinetic displacement characterization that also indicated at least two forms of soil phosphorus (169). Similarly, strontium or calcium showed at least two adsorption sites on four clays when

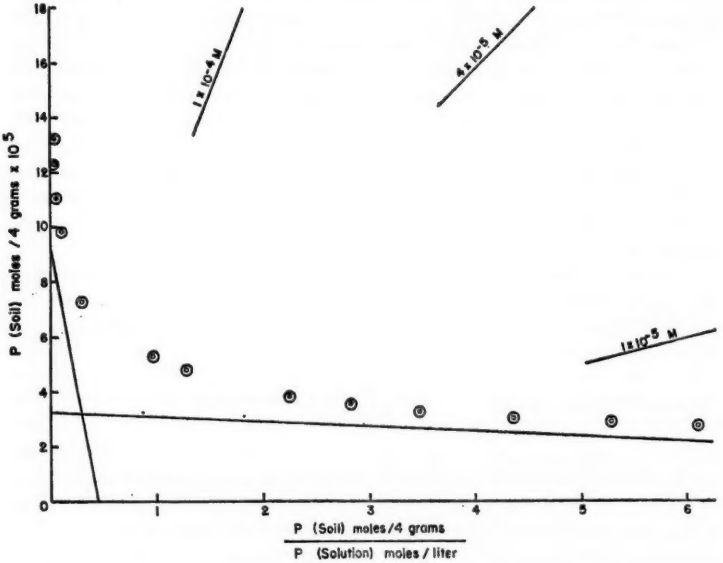


FIG. 1. The relationship between P(soil) and P(solution) where P(soil) is the amount of phosphorus adsorbed and P(solution) is the equilibrium phosphorus concentration. The slope of radial lines through the origin is the isoconcentration in solution. The three strikes on the periphery represent radial isoconcentration lines.

the system was characterized in terms of Equation 2 (84). Strontium was adsorbed more tightly than calcium on either site and was exchangeable with all other cations including sodium, barium, calcium, and yttrium. The constant ΣM(soil) in Equation 2 was essentially equal to the exchange capacity of the clay as determined by isotopic dilution or by leaching with HCl or NH_4OAc (69, 84). The apparent dissociation constant of the two sites differed by 2 to 3 orders of magnitude both for strontium and for calcium.

The mass action equation characterization of M(soil)-M(solution) relations is comparable to the description afforded by a Langmuir isotherm. Indeed, Hitchcock (88) has indicated the formal identity of Langmuir's adsorp-

tion equation with the law of mass action. Both equations involve a capacity constant and a constant related to the energy of retention.

When the constants are known, a knowledge of per cent saturation permits calculation both of the ion concentration and also of the ability of the soil to maintain this concentration under stress; a knowledge of the ion concentration permits a calculation of the per cent saturation and the consequent ability of the soil to maintain the ion concentration under stress. Thus, the yields and mineral composition of several plant species were shown to be related to the exchangeable sodium percentage rather than to the absolute level of exchangeable sodium (10).

Identification vs. solid-solution relations.—The two approaches to the characterization of M(soil), identification of the solid phase and solid-phase —soil-solution relationships, are not necessarily incompatible (18, 62, 88, 116). There is, however, a real difference in utility when describing the soil in relation to the plant.

When the adsorption constants are known, the ion concentration M(solution) may be predicted both when the ion is removed from the system (as by plants) and when the ion is added to the system (as by fertilization). However, neither mineralogical identification nor solubility products are sufficient to predict changes in the soil-solution concentration of the nutrient ion M(solution) when the ion is added or removed. Dilution of a soil system with water or leaching of a nutrient ion invariably result in a decrease in the equilibrium concentration of the nutrient ion. Even if solubility products remain constant with different soil:soil-solution ratios, the concentration of the nutrient ion may vary (5). Furthermore, the mean activity of a nutrient ion complex in a soil system may even increase with added nutrient (35).

The solid phase is the important capacity factor continuously replenishing M(solution) according to the relationship $M(soil) \rightleftharpoons M(solution)$. Adsorption equations describe this relationship and permit predictions of the changing ion concentration during a season's growth. Mineralogical or chemical characterization of the solid phase is more useful in describing a reproducible characteristic of a given soil and may offer a better description of the long term supply of a nutrient element.

Movement to the Solution-Root Interface

The ion M(solution) must be continuously replenished at the solution-root interface by movement in solution. The rate of ion uptake by the root is governed by the concentration of ions and the ease of movement in solution (186). Movement in solution is particularly important when the solution concentration is low (185). If there is a concentration gradient in the bulk soil solution from the solid—solution-phase interface to the solution—root interface, then the relative proximity of the metabolizing root to the solid—solution-phase interface may alter ion uptake by affecting the concentration of M(vicinity of root). This may give the appearance of solid-phase effects (107, 130).

The replenishment of M(solution) at the solution—root interface may take place either by simple diffusion through the liquid phase or by bulk movement along with the water. Diffusion may be relatively more important over very short distances while bulk movement is effective over longer distances. There is no general agreement on the relative importance of these two mechanisms in supplying ions to the plant root.

Corn growing in the field uses a total of approximately 20 acre-inches of water (145). About half of this is lost by transpiration. A 100 bushel corn crop will utilize 25 to 50 pounds of phosphorus, 100 to 200 pounds of potassium, and intermediate amounts of calcium and magnesium (145). If the soil solution contains approximately 20 ppm of P (6×10^{-4} *M*), 100 ppm of K (2×10^{-3} *M*), 40 ppm of Ca (1×10^{-3} *M*), and 40 ppm Mg (1.7×10^{-3} *M*), then the 10 acre-inches of soil-solution water that is eventually transpired contains the high amounts of these elements needed by the plant. Under these conditions, diffusion of the element M (solution) is unimportant, and movement with the bulk of the water is the primary factor carrying the ion to the plant. This does not necessitate ion movement into the plant with the water. Ions and water move into the plant independently, reflecting entirely different physical-chemical forces; nevertheless, the water moving to the root carries ions in it. A portion of these ions is taken up by the plant by an independent mechanism. The remainder is left behind at the root surface or on the soil particles in the vicinity of the root where they represent a subsequent source of supply.

The data in Table I indicate that soil solution concentrations approaching the above levels or higher are general for most listed elements except phosphorus. When the concentration of an ion in the soil solution is lower, as for phosphorus, the other types of ion movement (diffusion, or movement in the soil with water under such forces as transpiration, gravitation, evaporation, or turbulence) may become more important (41, 113, 159, 167, 170).

The contribution of diffusion to ion movement in solid—solution systems has been discussed by several workers (130, 170, 185, 186). Although the diffusion coefficient for the various ions in pure solution is known, it is necessary to re-evaluate for a system in which the ion is adsorbed, some solid particles are charged, and the water films are not necessarily continuous. A step toward these corrections has been made in experiments using radioactive rubidium (117). The diffusion coefficient of rubidium was determined in the presence and absence of different sized glass beads. The results indicated a marked depression of diffusion rate by the beads themselves, involving at least a factor of two. The effect of charge and film continuity can only be estimated at present.

Soil water movement also transports ions to the root. A diffusion equation has been used to describe the movement of water at all moisture contents (73). Depending on the initial soil moisture content, the soil water diffusivity is 5×10^{2} to 5×10^{5} times greater than the apparent diffusion coefficient of phosphate ion in soil (170). Therefore, the rate of water movement should be

very much greater than the rate of ion movement by diffusion. If the movement of water is sufficiently rapid, then the diffusion of ions under a concentration gradient is overwhelmed by the movement of the liquid phase "hydrodynamics dispersion" (41).

Roots continuously grow into new soil—water regions and new moisture sources. Thus, there is a continuous flow of water to the soil—root boundary (113, 159). Shapiro, Armiger & Fried (170) showed that diffusion did not limit the phosphorus supply to corn and soybeans because of the offsetting effect of the soil—water transport factor on ion renewal at the plant root.

The ion M moving in the soil—water stream not only helps maintain the concentration of the ion in the solution at the root interface (intensity factor) but also helps maintain the level of adsorbed ion on the soil in the immediate vicinity of the root (capacity factor). Thus, the greater the amount of water movement in the vicinity of the root, the higher the mean concentration of the ion at the root interface during the growing season. In other words, the greater this movement, the larger the soil volume that contributes to maintaining the concentration of the ion M in the vicinity of the root.

THE PLANT SYSTEM

Entrance Into Root

Ions in the soil solution bathing the plant roots enter the root. This entrance into the root has been described as both an active and passive process.

Passive entry.—Passive entry is supported by the work of Hylmo and his associates (102, 103), Epstein (55, 56), Wright & Barton (191), Kramer (118), and Kylin & Hylmo (122). This envisions a continuation of the soil solution into the root in the so-called "outer" or "free" space (55). There is considerable evidence that apparent "free" space exists in biological material (11, 19, 122, 125) but not necessarily conclusive evidence that living plant roots have appreciable space to which both cations and anions have free and reversible access from the soil solution.

The relative importance of free space in ion accumulation in roots relates to its possible effect on the concentration of M(vicinity of root) in Equation 1. Donnan effects, which might conceivably change the effective concentration of monovalent and divalent ions in a predictable pattern, do not appear to affect the ion concentrations appreciably (71, 111, 124, 172, 179). The concentration of M(vicinity of root) would also be different if diffusion into the root "free" space was rate limiting to the accumulation process. If diffusion were rate limiting, ion uptake should be directly proportional to concentration. Generally, it is not. The high temperature coefficients of ion accumulation for monovalent cations (89, 106), monovalent anions (95, 97, 106), and phosphate (94, 95), also indicate that diffusion is not rate limiting for these ions. The diffusivity of monovalent cations and anions is not very different from that of such divalent ions as calcium and sulfate (181). Since the turnover rates of calcium and sulfate are much slower than for monovalent cat-

ions and anions (46, 71, 111), diffusion is even less likely to be rate limiting for calcium and sulfate. Furthermore, diffusion effects are likely to be more pronounced the lower the concentration of the ion M(vicinity of root). Of the major nutrient elements, phosphorus occurs in solution at the lowest concentration. Yet, Jackson & Hagen (104) found that at $10^{-5}M$ phosphate, the rate of ^{32}P appearance was constant in the five major products of phosphate uptake starting at 15 sec. and continuing for at least 40 min., indicating that new sites of incorporation were not becoming available as diffusion time increased. Thus, if "free" space does exist in living plant roots to any appreciable extent, it does not appear to affect the concentration of M(vicinity of root).

Active entry.—Active entry is supported by the work of Brouwer and his associates (20, 21), van den Honert and his associates (97, 98), and others (72, 162, 164). Van den Honert in 1937 (96) envisioned phosphate as, "adsorbed by the surface layer of the protoplasm of the root cells and subsequently carried on by a mechanism resembling a constantly rotating belt conveyer, which moves its charge from the surface depositing it inside and returning empty to be charged again." He visualized the conveyer mechanism as the active process. Active entry is further suggested by the rapidity of response to the outside solution by the accumulating mechanism of the root. Thus, steady-state uptake for the monovalent cations, monovalent anions, and phosphate is reached in less than five minutes (58, 71, 79), and inhibitor effects on active accumulation are essentially instantaneous.

Active Accumulation

General theory.—Whether ions are actively accumulated at or near the root surface or move through free space before active accumulation occurs, active accumulation finally occurs in the cells of the root. From van den Honert's conveyer belt picture (96) through models of Osterhout (152), Rosenberg (161), Jacobson *et al.* (105), Epstein & Hagen (58), and Florell (64), a carrier process for this step has been variously described. Fried *et al.* (72), following Epstein & Hagen (58), used the following notation:

$$R + M \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} MR \qquad 3.$$

$$MR \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} M\ (\text{Accumulated}) + R' \qquad 4.$$

where M is the ion, R the carrier containing a specific binding site, and MR an intermediate that goes through an active process resulting in the deposition of the ion across an energy barrier. Equation 3 describes the partial saturation of the binding site. Equation 4 requires metabolism and is therefore the active accumulation step. It is rate limiting and essentially irreversible insofar as active ion accumulation is concerned.

If Equations 3 and 4 or a variation of these equations describes ion ac-

cumulation, the mathematical expression of these equations must quantitatively describe the process. Thus, the following equation must hold:

$$v = \mathrm{MR}\, k_2 t \qquad 5.$$

where v is the amount of ion actively accumulated and k_2 is a constant. Where the concentration of M in solution is maintained essentially constant and the plant root maintains a constant carrier concentration, then a plot of v vs. t should give a straight line (steady state). This has been found experimentally for many ions (22, 58, 59, 71, 78, 127). If any of the various chemical equation descriptions of the carrier mechanism is valid, then ion uptake is subject to mathematical analysis. Ion uptake reflects either rate constants or the concentration of reactants such as carrier concentration; changes in ion uptake must also reflect changes in these factors.

Biological constants.—The uptake of a given ion species can be described essentially by three constants, ΣR, k_2, and K_m, where ΣR is the total concentration of effective carrier and (from Equation 5) equals MR when the carrier site is saturated; i.e., $v = V_m$, K_m is the Michaelis-Menten constant (137), an apparent dissociation constant of MR, the ion-carrier combination; and k_2 is the turnover constant in Equation 4 (147).

(*a*) *Relation to the soil solution.*—Tables II and III summarize Michaelis-Menten constants determined for plant roots by various investigators. Table II gives results obtained with anions and Table III with cations.

TABLE II

MICHAELIS-MENTEN CONSTANTS OF ANION CARRIER COMPLEXES IN PLANT ROOTS

Ion Species	Plant Species	K_m		Reference
		At Relatively High Concens.	At Relatively Low Concens.†	
Phosphate	Barley	0.4×10^{-4}	1×10^{-6}	Hagen & Hopkins (78)
Phosphate	Barley	3×10^{-4}	3×10^{-6}	Hagen *et al.* (79)
Phosphate	Barley	9×10^{-4}	3×10^{-6}	Noggle & Fried (147)
Phosphate	Alfalfa	9×10^{-4}	3×10^{-6}	Noggle & Fried (147)
Phosphate	Millet	10×10^{-4}	4×10^{-6}	Noggle & Fried (147)
Phosphate	Sugar cane	—	1×10^{-6}*	van den Honert (94)
Sulfate	Barley	—	10×10^{-6}	Leggett & Epstein (127)
Bromide	Barley	13×10^{-3}	—	Epstein (54)
Bromide	Barley	10×10^{-3}	20×10^{-6}	Hagen *et al.* (see text)
Nitrate	Corn	—	20×10^{-6}	van den Honert & Hooymans (97)
Iodide	Wheat	3×10^{-3}*	—	Böszörményi & Cseh (16)

* Estimated from the data.

† If phosphate ion is $HPO_4^{=}$ (78) multiply by 0.00064 to obtain K_m.

TABLE III

MICHAELIS-MENTEN CONSTANTS OF CATION CARRIER COMPLEXES IN PLANT ROOTS

Ion Species	Species	K_m		Reference
		At Relatively High Concens.	At Relatively Low Concens.	
Rb	Barley	4×10^{-3}		Epstein & Hagen (58)
Rb	Barley	2×10^{-3}	4×10^{-6}	Fried & Noggle (70)
K	Barley	1×10^{-3}	3×10^{-6}	Fried & Noggle (70)
K	Corn	0.2×10^{-3}	2×10^{-6}	Bange (7)
K	Barley	5×10^{-3}*		Overstreet *et al.* (153)
K	Corn	0.3×10^{-3}		Hanson & Kahn (80)
K	Soybeans	0.5×10^{-3}		Kahn & Hanson (111)
Cs	Barley	5×10^{-3}*	8×10^{-6}	Bange & Overstreet (8)
Na	Corn	3×10^{-3}		Huffaker & Wallace (99)
Na	Soybeans	2×10^{-3}		Huffaker & Wallace (99)
Na	Radish	1×10^{-3}		Huffaker & Wallace (99)
Na	Barley	4×10^{-3}	10×10^{-6}	Fried & Noggle (70)
Sr	Barley		60×10^{-6}	Epstein & Leggett (59)
Sr	Barley	3×10^{-3}	3×10^{-6}	Fried & Noggle (70)
Li	Barley	6×10^{-3}*		Epstein (57)

* Estimated from the data.

The results in Tables II and III show how the soil solution concentration and plant uptake are related. Thus at a $10^{-6}M$ concentration of phosphate [$10^{-9}M$ concentration of $HPO_4^{=}$, if the interpretation of Hagen & Hopkins (78) is correct] the carrier site effective at lower phosphate concentrations is half saturated. Increasing phosphate concentration in the soil solution cannot increase phosphate accumulation more than twofold until the site effective at higher concentrations of phosphate, [$H_2PO_4^-$ according to Hagen & Hopkins (78)] becomes effective. Table I (p. 93) indicates that in acid soils it is not uncommon for the concentration of phosphate to be about as large as the K_m of the site effective at lower concentrations, i.e. $10^{-6}M$. The other major nutrient anions, sulfate and nitrate, are less well defined both as to concentrations in the soil solution and Michaelis-Menten constants. There is a good indication that at least two uptake sites occur with each anion. The two sites for phosphate are shown in Table II. In addition, Hagen, Leggett & Jackson, at Beltsville (unpublished), have determined the K_m's at both sites for bromide; Böszörményi (16) noted two sites in chloride and iodide uptake by wheat roots; and Leggett & Epstein (127) noted a second concentration function for sulfate on barley roots.

The concentration of cations in the soil solution (Table I) indicates that the site effective at the lower concentration is essentially saturated. However, the site that is effective at higher concentrations is generally at a lower de-

gree of per cent saturation. Thus, assuming similar carrier concentrations and turnover constants at both sites, most of the uptake is from the site with the low K_m. Increasing the concentration by fertilization is effective through an increase in the concentration and a consequent turnover of the MR of the site with the higher K_m.

(*b*) *Relation to the plant.*—It is an oversimplification to consider K_m alone without the other biological constants in the ion absorption process, ΣR (carrier concentration) and k_2. The data on these latter two constants are limited, but tentatively suggest that for a given ion the carrier concentration of the site effective at higher concentrations is from five to ten times as great as the site that is effective at lower concentrations (71, 79, 147). The carrier concentration of different cations appears similar in magnitude and the k_2 for the same ion appears similar at both sites (71). Hagen *et al.* (79) were able to detect that the site effective at lower concentrations of phosphate has twice the k_2 of the other phosphate site. The k_2 for different monovalent cations was similar, and at least twelve times as great as that found for strontium (71). This is consistent with the observed relatively slower turnover of divalent cations by plant roots (58, 59, 123).

A comparison between species indicates a marked similarity in the order of magnitude of the K_m even between investigators using different techniques (Tables II and III). The difference in rate of uptake of phosphorus by different plant species was shown to be primarily attributable to as much as 25-fold differences in site concentration as compared to two- or threefold differences in K_m or k_2 (147).

Ionic interactions.—Many of the interactions between ions may be more specifically evaluated by considering the effect of one ion on the carrier uptake mechanism of another ion. Stimulation and inhibition effects, including uncompetitive and noncompetitive (58), and changes in turnover rate, manifest themselves through changes in the biological constants ΣR, K_m, and k_2. Competitive inhibition in ion uptake is the decrease in uptake of one ion due to the affinity of another ion for the same binding site on the carrier. Competitive inhibition only changes the effective concentration of the ion carrier complex, MR, of the ion under study.

While the K_m can be estimated over a limited concentration range from uptake-concentration curves, the number of sites or the nature of the stimulation or inhibition is not as readily evident unless a different type of graphical representation of the mathematical analysis is made.

(*a*) *Graphical analysis.*—Two types of plots have been used on ion uptake data to determine biological constants. These are the double reciprocal plot; i.e., $1/v$ vs. $1/\mathrm{M}$ from the equation

$$\frac{1}{v} = \frac{1}{V_m} + \frac{1}{\mathrm{M}} \cdot \frac{K_m}{V_m}$$

(129); or v vs. v/M plot, a result of a mathematical rearrangement of the same equation [namely, $v = -K_m v/\mathrm{M} + V_m$ (92)] where v is the amount of ion ac-

cumulated in a given time and V_m is the amount accumulated when the carrier is completely saturated with M, i.e. $MR = \Sigma R$. Both types of plots are reliable and the relative merits of each have been discussed in relation to enzyme analysis (47, 49, 51, 93).

The two types of plots are illustrated for the same data in Figures 2 and

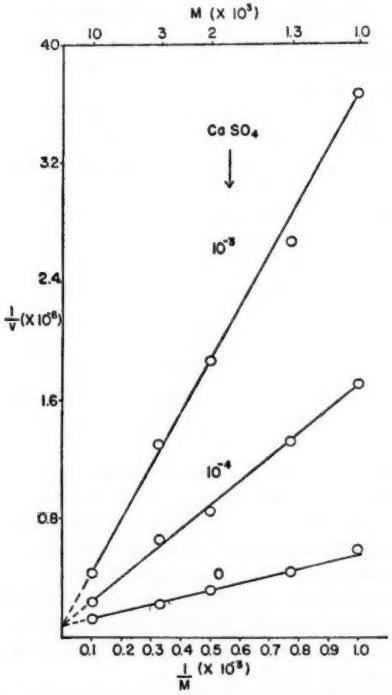


FIG. 2. Inhibition of lithium absorption by calcium (57) plotted in the form preferred by Dixon & Webb, $1/v$ vs. $1/S$ (47).

3, the double reciprocal plot in Figure 2, and the v vs. v/M plot in Figure 3. Figure 2 is taken from the literature regarding an experiment to test the nature of the inhibition of lithium uptake by Ca (57). The conclusion reached by the author was that Ca and Li are competitive. While this seemed reasonable since the straight lines drawn through the several points appear to satisfy the condition of changing slope with constant intercept, Figure 3, a replot of the same data, illustrates the hazard of this extended extrapolation. For these particular experimental conditions a v vs. v/M plot appears prefer-

able. This set of data contained two concentrations of inhibitor and a different conclusion is readily evident when the K_i, an inhibition constant, is calculated from each concentration of inhibitor. The K_i varies approximately fourfold with a tenfold change in inhibitor concentration whereas the K_i should theoretically remain constant for competitive inhibition. Not all competitive inhibition data contain this double check (100).

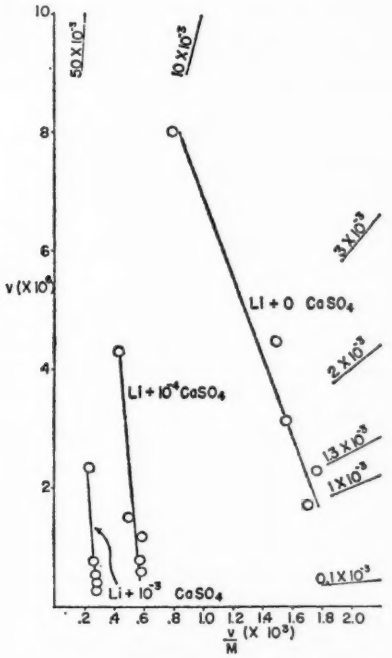


FIG. 3. Same data as in Figure 2, plotted in the form preferred by Hofstee, v vs. v/S (92).

Dixon & Webb (47) state that "our main reason for preferring the double reciprocal plot is that one can readily identify the different points with particular substrate concentrations, and so see what is taking place." Although the concentrations are not very evident in either type of plot, this is easily remedied. Actual concentrations may be shown on an inverse axis on the double reciprocal plot (see Figure 2), and as strikes around the edge representing the isoconcentration lines that radiate from the origin on the v vs. v/M plot (see Figures 1 and 3).

(*b*) *Competitive inhibition.*—Competitive inhibition between ions in the ion uptake process by plants has been determined. Thus, K^+, Cs^+, and Rb^+ are competitive (8, 36, 58, 136); Ca^{++}, Sr^{++}, and Ba^{++} appear competitive (59); $SO_4^=$ and $SeO_4^=$ are competitive (127); Cl^-, Br^-, and I^- appear competitive (16, 53); as do phosphate and arsenate (Hagen & Hopkins, unpublished). Hydrogen appears to compete for all sites that bind cations (70, 146), and hydroxyl may compete for all sites involving anions (78, 105). Sodium competition for potassium is not clear. There is some indication that sodium may compete with one of the two potassium sites but not with the other (7). No two nutrient ions have ever been shown to utilize the same binding site of ion uptake when the strict definition of competitive inhibition is maintained.

TABLE IV

COMPETITIVE INHIBITION CONSTANTS, K_i, FOR ION UPTAKE BY BARLEY ROOTS

Sorbed Species	Ion K_m	Competing Species	Ion K_i	Reference
Na	1×10^{-5}	H	2×10^{-6}	Fried & Noggle (70)
K	3×10^{-6}	H	1×10^{-5}	Fried & Noggle (70)
Rb	4×10^{-6}	H	1×10^{-5}	Fried & Noggle (70)
Sr	3×10^{-6}	H	1×10^{-5}	Fried & Noggle (70)
Rb	4×10^{-6}	K	4×10^{-6}	Fried & Noggle (70)
Rb	3×10^{-3}	K	4×10^{-3}	Epstein & Hagen (58)
Rb	5×10^{-3}	Cs	9×10^{-3}	Epstein & Hagen (58)
Sr	60×10^{-6}	Ca	30×10^{-6}	Epstein & Leggett (59)
Sr	60×10^{-6}	Ba	80×10^{-6}	Epstein & Leggett (59)
Br	13×10^{-3}	Cl	13×10^{-3}	Epstein (54)
So_4	10×10^{-6}	SeO_4	7×10^{-6}	Leggett & Epstein (127)
H_2PO_4	4×10^{-5}	OH	2.5×10^{-9}	Hagen & Hopkins (78)
HPO_4	5.5×10^{-10}	OH	2×10^{-11}	Hagen & Hopkins (78)

A possible indication of two nutrient ions occupying the same site is the slight suggestion that NH_4 and Cs are competitive (8), assuming the Cs site represented was identical to the potassium site.

Competitive inhibition constants K_i's are recorded in Table IV. The results show the quantitative relationship between competing ion pairs. Knowing the composition of M(solution), the effect of a competing ion of a given concentration can be estimated. Thus, one of the effects of a change in soil pH is not only an effect on the concentration of M(solution) due to soil effects, but an effect on the concentration of M(absorbed) due to competitive inhibition of hydrogen and hydroxyl ions for cations and anions.

(*c*) *Other ionic interactions.*—Other inhibition or stimulation effects of one ion on the uptake of another also may be related to the ion uptake mechanism as manifested by changes in the biological constants ΣR, K_m, and k_2. An approach to this type relation was made by Hagen *et al.* (79) for phosphate ab-

sorption by barley roots. Kahn & Hanson (111) utilized the effect on biological constants to explain the effects of calcium on the uptake of potassium in soybeans and corn roots.

Many investigators have suggested nonspecific effects on the ion uptake process such as solid-phase effects (107, 130) and effects due to the "so-called" cation exchange capacity of roots (48, 114, 135, 138, 173, 188). Solid-phase effects, as a mechanism for moving ions from soil particles to plant roots [i.e., M(soil)$\rightleftharpoons$M(absorbed)], are less likely to be of significance if the occurrence of specific carrier sites for specific ions is the mechanism of ion uptake. Cation exchange capacity of roots represents a nonspecific exchange process with predictable interactions such as Donnan effects and lyotropic series relationships. The specificity of the ion uptake mechanism for individual ions, the lack of demonstratable Donnan effects, and the inability of divalent cations to necessarily decrease the uptake of monovalent cations at equal ionic concentrations suggest that no nonspecific cation exchange capacity of roots is directly involved in the ion uptake process. An adsorption process that is specific for the ion and carrier as indicated in Equation 3 probably does take place.

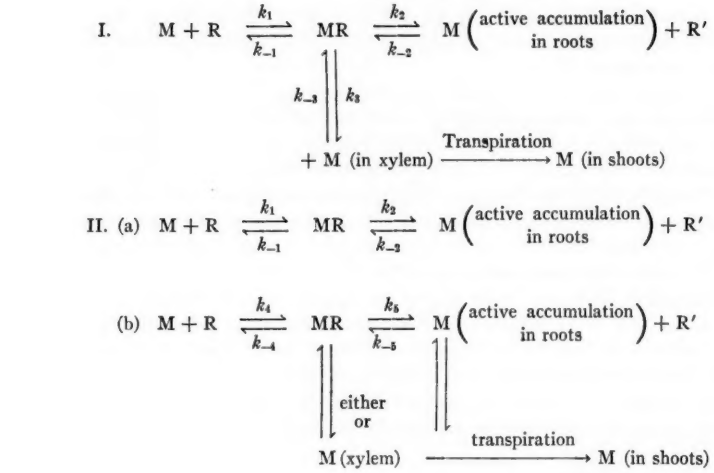
Movement to Tops

Ion accumulation in growing plants does not stop with ion accumulation in roots but includes movement to the shoots or tops. There is considerable evidence that the ions moving to the top are not necessarily those ions accumulated by the roots (27, 55, 72, 184). Ions are generally presumed to move to shoots primarily in the xylem; however, there is a difference of opinion as to whether a prior active step is a necessary part of this transport process.

Passive.—Passive uptake in its simplest form is a continuation of the soil solution through the "free" space of the plant in both roots and shoots. Hylmo and his associates (102, 103), Epstein (55, 56), Kramer (118), Kylin (121, 122), and Pettersson (155) have suggested that an appreciable amount of ion transport to shoots occurs in this manner. However, other investigators such as Brouwer (21), Russell & Shorrocks (163, 164), Fried *et al.* (72), and Russell & Barber (165) have concluded that a prior active step is necessary before the ion can reach the xylem.

Passive uptake suggests a concentration function in which uptake to shoots is directly proportional to concentration, and relatively independent of the presence of other ions, temperature, and other metabolic inhibitors. A prior active step suggests a concentration function reflecting the biological constants ΣR, K_m, and k_2, and is responsive to competitive and metabolic inhibitors and changes in temperature.

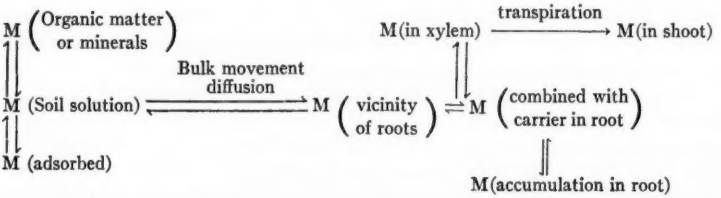
Active.—Fried *et al.* (72) concluded that a prior active step was a necessary component of ion transport to shoots and related the active step to a carrier mechanism involving the biological constants ΣR, K_m, and k_2. Their two suggested chemical descriptions of the process, consistent with their data, are given below as I and II.



Description I represents two active metabolic steps competing for the same carrier-ion intermediate; one resulting in root accumulation and the other in shoot accumulation. Description II represents two entirely separate active processes, one leading wholly to root accumulation and the other primarily to shoot accumulation. Fried *et al.* state that "since description I places more restrictions on the system, it is more useful until proven inconsistent with further experimental data."

CONCLUSION

The overall picture of ion uptake in the soil-plant system is summarized in the following equation:



where the source of the ion is organic matter, minerals, or surface adsorbed ions. For surface adsorbed ions, the concentration function can be described by a capacity factor and an apparent dissociation constant. The rate of dissolution of the ions is apparently so rapid that the ions in solution are in equilibrium with the ions on the surface. The ion in the soil solution then moves to the root by bulk movement with the water and by diffusion. At the root surface, the ion combines with the carrier and an active turnover takes

place, resulting in either ion accumulation in the root or deposition of the ion into the upward transport system, the xylem. Finally, the ion may be utilized by the living cells. These active processes are best described by such biological constants as K_m, the apparent dissociation constant of the carrier-ion combination; R, the site concentration of the specific carrier; and k_2, the rate of turnover of the carrier-ion combination.

The position of the rate limiting step, or steps, in the overall process is not known for all environmental conditions and plant species. It can conceivably be during movement of the ion to the vicinity of the plant root, at the turnover of the carrier-ion combination, or at the transport step to the plant top (transpiration).

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PROTEIN SYNTHESIS[1,2,3]

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In recent years, the mechanism of protein synthesis has been studied by a steadily increasing number of investigators. An examination of the resulting literature gives impressive evidence that much information has been collected on many phases of the process of protein synthesis and that a picture is emerging of the chemical steps involved in the synthesis of at least some kinds of proteins. One also is impressed, however, with the staggering complexity of detail in the protein synthesis mechanism, with the shaky experimental foundations on which many of our current theories still rest, and with the magnitude of the research effort that will be necessary before we can state confidently that we understand how various protein molecules are synthesized. For example, protein synthesis apparently requires: 20 different amino acid-activating enzymes; at least 20 separate kinds of sRNA; a complex aligning mechanism containing some sort of genetically-controlled code; and various additional enzymes for peptide bond formation, release of the completed protein from the "template," and reactivation of the sRNA. As yet, none of the activating enzymes has been isolated in pure form; promising methods for the separation of different sRNA molecules have only just been developed; the "coding problem" appears to be getting more complex; and the existence of enzymes catalyzing peptide bond formation and the release of completed proteins has only been recognized during the past year. However, all of these components of the protein synthesis process are under intensive investigation, and their manner of operation is steadily being clarified.

This review will assess the present state of our knowledge concerning the chemical steps involved in protein synthesis and suggest some (probably obvious) courses for future investigation. Many excellent reviews on protein synthesis have appeared recently, expressing a variety of viewpoints. The reviews of Dalgliesh (1), Crick (2), Bonner (3), Koshland (4), Simkin (5), Hoagland (6), Campbell (7, 131), and Medvedev (8) are especially recommended because each presents useful ideas in addition to reviewing one or more phases of the subject. A most comprehensive and valuable review on protein synthesis is to be found in the report of the *International Symposium on Protein Biosynthesis* (9).

[1] The survey of literature pertaining to this review was concluded in August 1960.

[2] The preparation of this review, and the studies of the author and his colleagues referred to herein, have been made possible by grants from the Ohio State University (through the Council on Research) and the Public Health Service (C-3725). The excellent assistance of Sandra Whitman is gratefully acknowledged.

[3] Abbreviations used: GDP (guanosine diphosphate); GMP (guanosine monophosphate); GTP (guanosine triphosphate); sRNA (amino acid-acceptor RNA).

Enzymatic Synthesis of Protein

Net protein synthesis by cell-free preparations.—An important difficulty in the study of the mechanism of protein synthesis is the failure of many cell-free preparations to catalyze a measurable net synthesis of protein. This has forced investigators to employ either "amino acid incorporation into protein" or the increase in activity of some enzyme as measures of protein synthesis. Because neither of these methods necessarily measures actual protein synthesis, results obtained by their use are generally interpreted with caution. It is heartening, therefore, that a number of investigators have

TABLE I

Protein Synthesis by Cell-Free Systems

Preparation and Reference	Conditions	Protein Formed*	Protein Formed Per Min.*
Bacillus megaterium—disrupted protoplasts (10)	180 min.-30°C.	2580.0	14.33
Pea—ribosomes (11)	30 min.-38°C.	360.0	12.00
Neurospora crassa—particles (12)	60 min.-30°C.	219.0	3.65
Alcaligenes faecalis—particles (13)	60 min.-30°C.	211.0	3.52
Rat liver—large particles (14)	30 min.-39°C.	105.0	3.50
Pea—ribosomes (15)	90 min.-37°C.	250.0	2.78
Tetrahymena pyriformis—kinetosomes (16)	90 min.- —	242.0	2.69
Staphylococcus aureus—disrupted cells (17)	90 min.-37°C.	186.0	2.07
Pigeon pancreas—large particles (14)	30 min.-39°C.	50.0	1.67
Rat liver—small particles (14)	30 min.-39°C.	38.0	1.27

* μg. protein formed by 1 mg. of preparation.

now isolated preparations that will catalyze measurable protein synthesis. The preparations currently available and their calculated rates of protein synthesis are listed in Table I. Many of these preparations have been characterized but little, and further characterization will be of considerable value. This would appear to be especially true of disrupted protoplasts of *Bacillus megaterium*, which have been reported (10) to exhibit such impressive rates of protein synthesis. One would like to be certain, for example, that the preparations are free of intact protoplasts. A similar preparation has been isolated from disrupted protoplasts of *Escherichia coli* (18, 19), although information on the rate of protein synthesis has not appeared.

At present, pea ribosomes appear to be fairly typical catalytic agents for the net synthesis of relatively large quantities of protein (11, 15, 20). When ribosomes, isolated from three-day-old pea seedlings, are incubated with a

mixture of the 20 amino acids and amides normally found in protein, ATP, GTP, phosphoglycerate or phosphoenolpyruvate, KCl, $MgCl_2$, $MnCl_2$, and sRNA, each mg. of ribosomal protein catalyzes the formation of approximately 12 μg. of protein per min. (11). If a mixture of radioactive amino acids is used in this reaction, the newly-formed protein (which is found in the medium surrounding the ribosomes; the ribosomes themselves do not increase in protein) has a specific activity approximately the same as the mixture of free amino acids. This shows clearly that the amino acids are being converted into the protein. The protein formed by pea ribosomes has been characterized to some extent (11, 20). It is heterogeneous, and the various molecular species have molecular weights ranging from about 7500 to about 50,000. The protein exhibits a number of electrophoretic components, but these components do not migrate in the same fashion as the total nonparticulate protein of pea seedling cells. Most important, however, is the fact that the synthesized protein possesses a number of enzymatic activities. These include: adenosinetriphosphatase, phosphoglucomutase, aldolase, and pyruvic kinase. The enzyme activities increase in the same manner as the total protein being formed by the ribosomes. The possession of enzymatic activity by the protein formed by the pea ribosomes indicates that the ribosomes are assembling amino acids in some meaningful sequence, and are not merely forming polypeptide material. Protein synthesis by pea ribosomes is influenced greatly by the nature and concentrations of the components of the reaction system (11, 15). If the pea ribosome system is typical, it will be necessary for investigators using other systems to establish carefully the optimal concentrations of the various components of the reaction mixture in order to obtain maximal protein synthesis.

Two other promising experimental systems should be mentioned, although the amount of actual protein they have been reported to form is almost vanishingly small. Schweet *et al.* (21) have observed that rabbit reticulocyte ribosomes are able to incorporate radioactive amino acids into hemoglobin, and they conclude that the ribosomes are synthesizing hemoglobin. This would appear to be the case, but one can estimate from the data given (21) that 100 mg. of ribosomes (plus activating enzymes) are forming only 20 μg. of hemoglobin in 60 min., or 0.0033 μg. hemoglobin formed per min. by 1 mg. of total catalytic protein. However, apparently 85 per cent of the protein formed by the reticulocyte ribosomes is hemoglobin, so the synthesis of a single kind of protein can be studied with this system. If the system can be induced to form greater amounts of hemoglobin, it will be of great value in studies on the mechanism of protein synthesis. Likewise, Bates *et al.* (22) have reported a 20 per cent increase in cytochrome-*c* when calf heart mitochondria are incubated for 12 hr. with an amino acid mixture and an energy source. This would seem to be an extremely low rate of synthesis, but the system is potentially useful for studying mitochondrial protein synthesis and for studying the formation of a specific protein. Mitochondria are extremely complex structures, however, and it would not appear that this system can

be very useful in elucidating the mechanism of cytochrome-*c* synthesis until the cytochrome-forming structures can be isolated from the mitochondria.

From the results obtained thus far, one can expect not only that preparations catalyzing net protein synthesis will contribute to an increasing extent to our understanding of the mechanism of protein synthesis, but also that still more useful systems will become available.

Amino acid incorporation into protein.—Many cell-free preparations that are unable to catalyze a net synthesis of protein are, nevertheless, able to incorporate amino acids into protein. Although some doubt has been expressed in earlier reviews concerning the relevance of amino acid incorporation to the problem of protein synthesis, it now seems clear that amino acid incorporation into particulate protein represents a partial synthesis of protein molecules. The process being measured is apparently the addition of amino acids to the ends of growing polypeptide chains that are being formed on the surface of ribosomes or other particulate elements. Therefore, amino acid incorporation, in the absence of net protein synthesis, can give us information on those processes resulting in the assembly of amino acids on the ribosome or other particle, but can give no information on the completion of the polypeptide chain, its release from the catalytic surface, or possible subsequent folding of the polypeptide or its association with other completed polypeptide chains to form a biologically active protein.

One would like very much to know why some preparations (such as those listed in Table I) can synthesize proteins, while others, although capable of amino acid incorporation, cannot complete the synthesis process. For example, the ribosomal particles of liver (24) and reticulocytes (25) appear from *in vivo* studies to be important cellular sites for the formation of "soluble" proteins. The liver particles, *in vivo*, form serum albumin (26) while the reticulocyte particles form hemoglobin (27). In contrast, while isolated reticulocyte ribosomes seem capable of hemoglobin formation (21), liver microsomes or ribosomes are able to form only very limited quantities of protein (28, 29, 39, 131). Lingrel (28) has examined the relative inability of rat liver microsomes to form proteins. This failure does not seem attributable to some simple environmental condition (pH, substrate concentrations, lack of some factor, etc.), to the inactivation of the incorporating system, or to the failure of one or more amino acids to be incorporated into the forming polypeptide chains. The major difficulty appears to be the failure of the microsomes to release completed polypeptide chains. Lingrel (28) has calculated that the amount of amino acid incorporated by isolated rat liver microsomes is sufficient for the completion of a number of polypeptide chains. He has further shown that microsomes, previously labeled *in vivo*, release insignificant amounts of completed chains when the microsomes are subsequently incubated *in vitro*. These findings are in agreement with the observations of Campbell *et al.* (29) and Ogata *et al.* (39) that isolated rat liver microsomes will incorporate amino acids into recognizable serum albumin, most of which remains attached to the microsomal particles. Their data in-

dicate that the microsomes are completing serum albumin molecules, but are releasing only limited amounts from the catalytic surface. The same situation occurs if ribosomes are prepared from microsomes by solubilization of the reticulum proteins with deoxycholate in the presence of high concentrations of magnesium ions (28, 30, 31, 32). These ribosomes appear to catalyze the completion of polypeptide chains, but are not able to release significant quantities of the completed molecules (28).

There appears to be no information available on how this release may be accomplished in these relatively inactive systems. Preparations that catalyze net protein synthesis appear to possess a specific "release enzyme" which performs this function, but little is known at present about this enzyme. Whether these inactive preparations have an inactive "release enzyme," or whether they employ a different mechanism is not known, but this problem deserves further investigation.

Apparent enzyme synthesis.—Many investigators have measured the increase in activity of some enzyme in a cell-free preparation, and have then claimed that this increase represents the synthesis of new enzyme molecules. In the absence of other evidence of a net synthesis of protein, such claims are without foundation. The pitfalls inherent in measuring an increase in enzyme activity are illustrated by recent studies on the apparent synthesis of amylase by cell-free preparations. It has been observed that cell-free preparations of pigeon pancreas exhibit an increase in amylase activity when they are incubated with an amino acid mixture and an energy source (33, 34, 35). The increase is abolished by chloramphenicol, *p*-fluorophenylalanine, and ribonuclease, all inhibitors of protein synthesis. However, it has now been shown (36, 37) that the increase in amylase activity in cell-free extracts of pigeon pancreas is caused by a release of amylase already present in the preparation in latent form. The amylase is released in an active form into the medium if the microsomes are incubated with any of a number of amino acids (37). The total amylase content does not change. These findings demonstrate not only that a mere increase in enzyme activity cannot be accepted as representing protein synthesis, but also that inhibition by so-called specific inhibitors of protein synthesis (chloramphenicol, etc.) is not necessarily indicative that protein is being synthesized.

The synthesis of an induced enzyme by a cell-free preparation has now been clearly demonstrated. Kameyama & Novelli (38) have studied the incorporation of C^{14}-leucine into β-galactosidase molecules, catalyzed by particles from *E. coli*. The β-galactosidase was precipitated by a specific antiserum. Here, the increase in enzyme activity and the appearance of labeled enzyme molecules are parallel.

Mechanism of Protein Synthesis

On the basis of a large number of experiments by many investigators, the following chemical steps can now be proposed to occur in the synthesis of many kinds of protein molecules:

Step 1. Amino acid activation.

$$\text{Amino acid} + \text{sRNA} + \text{ATP} \overset{Mg^{++}}{\rightleftharpoons} \text{Amino acid-sRNA} + \text{AMP} + \text{Pyrophosphate}$$

Step 2. Peptide bond formation.

$$n(\text{Amino acid-sRNA}) + \text{Ribosome} \overset{\text{GTP, } Mn^{++}}{\rightleftharpoons} \text{Polypeptide attached to ribosome} + n\ \text{sRNA}$$

Step 3. Release of polypeptide from ribosome.

$$\text{Polypeptide attached to ribosome} \xrightarrow{\text{ATP, } Mg^{++}} \text{Polypeptide} + \text{Ribosome}$$

The further modification of the completed polypeptide chain, after release from the ribosome, by folding or by combination with other polypeptide chains (by formation of —S—S— or other linkages) is probable, but nothing is known of these processes at present. Our knowledge is greatest for the first step, much less for the second, still less for the third, and nonexistent for any further steps.

Amino acid activation.—It is now well established that enzymes that catalyze the activation of amino acids occur in the cells of many different kinds of plants, animals, and microorganisms (40, 41, 42, 75). All L-amino acids that normally occur in proteins are activated by these enzymes (42 to 47). Little or no activation of D-amino acids occurs. An enzyme that activates D-alanine has been reported (48), but it probably participates in bacterial cell wall synthesis, not protein synthesis. Various amino acid analogues are also activated. In general, the activated analogues seem to be those that are incorporated into protein (49). The enzymes that activate various amino acids seem to vary considerably in their stabilities (43, 45, 46), and this has complicated their isolation and study.

Enzymes that specifically activate a number of different L-amino acids have been purified from various sources (Table II). There is no evidence that any of these enzymes has been isolated in pure form as yet. The further purification and characterization of these enzymes, as well as the isolation of the enzymes activating the other amino acids and amides found in proteins, remains an important problem. In addition to the synthesis of amino acid-sRNA, many of the activating enzymes catalyze either an amino acid-dependent exchange of pyrophosphate with ATP, or the synthesis of amino acid hydroxamate. These additional reactions apparently are not catalyzed by all activating enzymes, and there is no good reason to expect that they should be. Claims that amino acid activating enzymes are not present in preparations in which no pyrophosphate-ATP exchange could be observed are certainly not valid. In this connection, the reviewer would like to suggest that the term "amino acid activation" should apply to the synthesis of amino acid-sRNA, not to the amino acid-dependent pyrophosphate-ATP exchange. If an amino acid is attached to sRNA, it is in an "activated" form

TABLE II

REACTIONS CATALYZED BY PURIFIED AMINO ACID ACTIVATING ENZYMES

Activating Enzyme	Source and Reference	Reactions Catalyzed*		
		Amino Acid-sRNA Synthesis	Pyrophosphate-ATP Exchange	Amino Acid Hydroxamate Synthesis
Alanine	Pig liver (50)	+	+	+
	Rat liver (51)	+	+	?
Glycine	*P. fischeri* (52)	?	−	+
Isoleucine	*E. coli* (53)	+	+	?
Leucine	*E. coli* (53)	+	+	?
Methionine	*E. coli* (53)	+	+	?
	Yeast (53, 54)	+	+	+
Serine	Beef pancreas (55)	?	(+)	+
Threonine	Calf liver (56)	+	+	?
Tryptophan	Beef pancreas (57, 58)	+	+	+
Tyrosine	Yeast (59)	?	+	+
	Pig pancreas (60)	?	+	+
Valine	*E. coli* (53)	+	+	?

* + positive
(+) variable
− negative
? not reported

for protein synthesis, regardless of whether the activating enzyme catalyzes a pyrophosphate-ATP exchange.

Although no claim has appeared that an amino acid activating enzyme has been isolated in pure form, the availability of highly purified enzymes has allowed considerable insight to be gained concerning the nature of the activation process. In fact, far more detailed information is available on amino acid activation than on any other step in protein synthesis. There seems little doubt that the activation reactions studied thus far proceed in the following manner:

$$E + \text{Amino acid} + ATP \rightleftharpoons E\text{-Amino acid-AMP} + \text{Pyrophosphate}$$
$$E\text{-Amino acid-AMP} + sRNA \rightleftharpoons E + \text{Amino acid-sRNA} + AMP$$

The occurrence of an enzyme-bound amino acid-AMP, in which the amino acid carboxyl is linked to the AMP phosphate, is supported by pyrophosphate exchange reactions (40, 51, 54, 61, 62), by the synthesis of ATP from pyrophosphate and synthetic amino acid-AMP (62, 63, 64), by the transfer of oxygen-18 from the amino acid carboxyl to the AMP phosphate (65, 66), and by the actual isolation of stoichiometric quantities of enzyme-bound amino acid-AMP (67, 68). The equilibrium of the reaction for amino acid-

TABLE III

SPECIFICITY OF AMINO ACID ACTIVATING ENZYMES FOR sRNA

Activating Enzyme	Source and Reference	sRNA Utilized Actively	sRNA Utilized Poorly or Not at All
Alanine	Rat liver (73)	Rat liver *E. coli*	
Alanine	Pig liver cytoplasm (50)	Pig liver cytoplasm Pig muscle	Pig liver nuclei Calf liver Yeast Pea seed
Alanine	Pig liver nuclei (50)	Pig liver nuclei	Pig liver cytoplasm Pig muscle Calf liver Yeast Pea seed
Leucine	Ascites cells (71)	Ascites cells Rat liver Calf liver	Yeast
Leucine	Guinea pig liver (72)	Guinea pig liver Rat liver	Yeast
Valine	*E. coli* (74)	*E. coli*	*A. vinelandii* Rat liver Yeast

sRNA synthesis has been found to be 0.32 (69) and 0.7 (70) for two different amino acids under slightly differing conditions. Thus far, every activating enzyme isolated has been able to form amino acid-sRNA from only one specific amino acid. Likewise, each activating enzyme appears to utilize only ATP, but further studies on the degree of nucleotide specificity are needed. Needed even more are further studies on the utilization of different kinds of sRNA for amino acid activation. Some activating enzymes have been reported to attach their amino acid to sRNA obtained from a variety of organisms, while others appear to be highly specific for the sRNA from the same organism from which the activating enzyme was obtained (Table III). Whether these differences are of some significance cannot be stated with confidence until both activating enzymes and sRNA from different organisms have been isolated in a pure state.

Amino acid-acceptor RNA (sRNA).—Knowledge of the structure and characteristics of sRNA will aid greatly in understanding the properties and specificity of amino acid activating enzymes. More important, however, such knowledge should aid in understanding the manner in which amino acids are arranged in a particular sequence to form a particular protein. As will be discussed later, the most obvious mechanism for specifying the order of amino

acids in a forming polypeptide chain is by the sequential base-pairing of each sRNA molecule (with amino acid attached) with a particular sequence of bases in ribosomal RNA.

The properties of sRNA differ considerably from the properties of other kinds of RNA. The molecular weights of various sRNA preparations are generally less than 50,000. Preiss *et al.* (69) reported a molecular weight for sRNA from *E. coli* of 30,000 to 50,000. Similarly, Zillig *et al.* (76) report a sedimentation constant of 4.6S, and a calculated molecular weight of 35,000. On the basis of very precise measurements, Tissiéres (77) found *E. coli* sRNA to have a sedimentation constant of 3.51 to 4.65S, and has calculated an average molecular weight of 25,500. In contrast, Brown *et al.* (19) obtained a sedimentation constant of only 2.5S for *E. coli* sRNA. From this, they calculated a molecular weight of 15,500 on the basis of sedimentation and viscosity, or 17,500 on the basis of sedimentation and diffusion. The sedimentation constant of rat liver sRNA has been reported to be 1.85S (78). Thus, there is considerable variation in the sedimentation values and molecular weights obtained in these early studies on sRNA. Further studies are needed, not only on sRNA preparations from other sources, but also on purified sRNA preparations that are specific for a single amino acid.

A second characteristic of sRNA is the almost universal presence of a terminal adenyl residue at one end of the sRNA molecule (76, 79, 80), and the presence of a guanosine-5′-phosphate at the other end (76, 80). During amino acid activation, the amino acid is attached through an ester linkage, involving its carboxyl group, to either the 2′ or 3′ hydroxy of the ribose on the terminal adenyl residue (69, 71, 81). Very little is known about the sequence of nucleotides between the adenyl group on one end and the guanosine phosphate on the other end, except that the terminal adenyl group is followed by either one or two cytidyl groups (79).

The composition of the polynucleotide between the terminal nucleotides is being determined, however, and provides a third remarkable characteristic of sRNA—the presence in sRNA of significant quantities of unexpected purine or pyrimidine nucleotides. Dunn (82) reported that rat liver sRNA contains (in addition to adenine, guanine, cytosine, and uracil) the following components (in moles per 100 moles uridine): pseudouridine, 25; 5-methylcytosine, 10; 6-methylaminopurine, 8.1; 1-methylguanine, 3.3; 2-dimethylamino-6-hydroxypurine, 3.0; and 2-methylamino-6-hydroxypurine, 2.3. Dunn *et al.* (83) have determined the nucleotide composition of sRNA from *E. coli*. In each 100 nucleotide residues, they find approximately 20 adenyl residues, 32 guanyl residues, 29 cytidyl residues, 15 uridyl residues, 2.1 pseudouridyl residues, 1.1 thymine ribonucleotide residues, 0.3 2-methyladenyl residues, 0.1 6-methylaminopurine ribonucleotide residues, and 0.1 1-methylguanyl residues. The relatively high content of pseudouridine has also been noted in yeast sRNA, which contains 14 moles of pseudouridine per 100 moles of adenylic acid (84). A marked correlation has been observed between the pseudouridine content of yeast sRNA and its ability to combine

with leucine (85). The significance, if any, of the unusual nucleotide composition of sRNA will only be revealed by further comparative studies on the composition and nucleotide sequence of sRNA molecules specific for different amino acids and from different sources. Much can be learned from these studies.

An important step in the further understanding of the nature of sRNA has been the recent development of methods for the separation of sRNA molecules that are specific for different amino acids. Two methods appear to be most promising. Brown *et al.* (19) have employed the principle that diazonium compounds react readily with certain amino acids to form stable bonds. The reactive amino acids are tyrosine and histidine, at pH 7–8.5, and lysine, arginine, cysteine, tryptophan, and glycine at pH 9. A cross-linked polydiazostyrene was prepared by treatment of polyaminostyrene with nitrous acid. When amino acid-sRNA, containing one of the above amino acids is stirred with polydiazostyrene under appropriate conditions, the amino acid, with its specific sRNA attached, is bound to the polydiazostyrene. All other RNA is not bound, and can be discarded. The specific sRNA can then be released from the polydiazostyrene by hydrolysis of the amino acid-sRNA linkage at pH 10. In this way, Brown *et al.* (19) have isolated sRNA molecules that will only react with tyrosine or only with histidine. It would appear that the above seven sRNA molecules can be purified fairly easily by this method. Molecules of sRNA specific for other amino acids might be purified by this method if the appropriate activating enzymes can be induced to attach a tyrosyl peptide to the specific sRNA (tyrosylvaline to valine-specific sRNA, for example).

A second method, applicable to sRNA molecules specific for each amino acid, has been developed by Zamecnik *et al.* (86). This method depends on the fact that the presence of an amino acid in ester linkage on the 2′ or 3′ hydroxy of the ribose of the terminal adenosine of sRNA prevents the oxidation of these positions to aldehydes by periodate. The sRNA is first freed of any attached amino acids by incubation at pH 10. A single amino acid is then attached to its specific sRNA molecules in the mixture by using an appropriate activating enzyme. The sRNA is then treated with periodate. The resulting aldehyde groups of sRNA molecules that had no attached amino acid are coupled with 2-hydroxy-3-naphthoic hydrazide to form hydrazones. The hydrazones are then coupled with tetrazotized *o*-dianisidine to form a blue dye. The dye-bound sRNA can be separated from the amino acid-sRNA by fractional precipitation with *n*-propanol.

The logical consequence of the separation of the various specific sRNA molecules should be the determination of the nucleotide sequences (87, 88, 89) of molecules specific for different amino acids and different activating enzymes. From this, insight into the coding mechanism will very probably be obtained.

Polypeptide formation from amino acid-sRNA.—In contrast to our knowledge of amino acid activation, our knowledge of the next step in protein

synthesis (polypeptide formation from activated amino acids on the ribosome surface) is very small. The transfer of amino acids from sRNA to transient protein associated with liver microsomes was first observed by Hoagland *et al.* (78). The transfer required GTP, ATP, an ATP-generating system, and an enzyme fraction. The enzyme fraction can be separated from amino acid activating enzymes, and was initially found to consist of both a heat-stable and a heat-labile fraction (90). The heat-stable factor can be replaced by glutathione (32) and increased concentrations of ATP and GTP (93). Similar requirements for the transfer reaction have been noted with pea ribosomes (45). Under such conditions, pea ribosomes will catalyze the conversion of amino acids attached to sRNA into "soluble" protein at considerably greater rates than are obtained with an equivalent quantity of free amino acids (91). This finding strongly supports the idea that sRNA-bound amino acids are intermediates in protein synthesis. The enzyme responsible for the transfer of amino acids from sRNA to ribosome-bound polypeptide has been purified partially by several investigators (20, 32, 92, 93). In the presence of the purified "transfer enzyme," there is no longer any need for ATP or an ATP-generating system for transfer of amino acids from amino acid-sRNA to the polypeptide chains forming on either pea ribosomes (20) or reticulocyte ribosomes (92). In the pea ribosome system, the simpler requirements appear to be due to the removal (during purification of the "transfer enzyme") of an enzyme which degrades GTP. The requirement for ATP and a generating system for transfer in the liver microsome system is undoubtedly also due to the presence of phosphatase activity (28). Thus, the formation of a ribosome-bound polypeptide from amino acid-sRNA apparently requires only GTP, a divalent cation, glutathione, and the "transfer enzyme." The role of these components in the transfer reaction is not clear. Especially interesting is the manner in which GTP participates in the reaction. It has been shown (20) that GTP is degraded to a mixture of GDP and GMP during the transfer reaction, but little is known beyond this as yet, and further insight will probably depend on the isolation of a pure "transfer enzyme," and its use in appropriate exchange experiments.

Two other facts about the transfer reaction should be noted. First, amino acids appear to be added sequentially to the N-terminal end of a growing polypeptide chain on the surface of the ribosome (11, 94). If only a single amino acid is transferred, the transfer is reversible (45). Second, it has been observed that amino acid-sRNA is transiently associated with the RNA of the ribosome during the transfer of amino acid from the sRNA to the end of the forming polypeptide chain (95).

From these various observations, a gross picture of the mechanism of amino acid sequence specification and polypeptide formation can be deduced. It would appear that the RNA located at the surface of the ribosome (96) contains the information necessary for specifying the amino acid sequence of a particular protein. Presumably, the location of each amino acid is coded into a short (and probably overlapping) sequence of nucleotides in the RNA.

The sequences specifying the position of each amino acid may be such that only the sRNA attached to the correct amino acid contains a complimentary nucleotide sequence. When a collision occurs between the appropriate amino acid-sRNA and the complimentary nucleotide sequence on the ribosome, the most logical consequence would be a temporary pairing of the bases of the two nucleotide sequences (Fig. 1). If the base-pairing has placed the amino acid attached to the sRNA next to the N-terminal end of the forming polypeptide chain, the "transfer enzyme" would then catalyze the transfer of the amino acid from the sRNA to the free amino group of the polypeptide. It should be noted that the polypeptide chain would be expected to be formed to one side of the ribosomal RNA, because the perpendicular configuration of the base and ribose in a nucleotide would place each amino acid attached

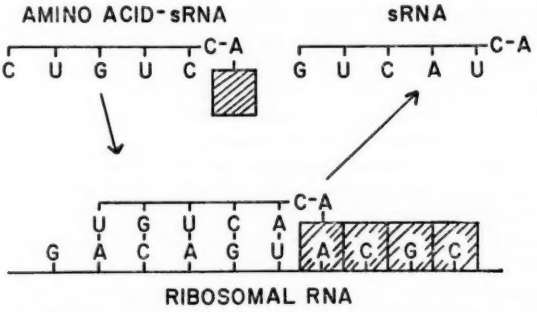

FIG. 1. Possible interaction of sRNA and ribosomal RNA during the transfer of amino acid from sRNA to the end of a growing polypeptide chain. The shaded squares represent amino acids and the sequences of letters represent RNA. A, G, C, and U represent adenine, guanine, cytosine, and uracil.

to sRNA to the side of the center line of a polynucleotide. It has been calculated (97) that sufficient collisions do occur between sRNA and ribosomes to allow amino acid transfer to proceed in the above manner.

In connection with this mechanism, one would like to know more about two details that are presently obscure. First, where and how does GTP participate in the process? Second, how is the sRNA released from the ribosome after it has transferred its amino acid to polypeptide; and how are molecules of sRNA, which contain no attached amino acid, prevented from "clogging-up" the ribosomal RNA? The obvious possible functions of GTP are in: the release of sRNA from the ribosome after amino acid transfer to polypeptide, the formation of a reactive intermediate during polypeptide formation, or the starting of a new polypeptide chain on the ribosome. It may well be that the entire transfer process is more complex than the scheme discussed above. Crick (98) has suggested that the "adaptor" (presumably, the base-

pairing) portion of sRNA may be covered by some secondary folding in the absence of an attached amino acid. This would prevent the base-pairing of an sRNA molecule, which had no attached amino acid, with the ribosomal RNA. It is possible that the folding operation might also result in the removal of an sRNA molecule from the ribosome after its amino acid had been transferred to polypeptide.

Release of completed polypeptide from the ribosome.—The existence of a separate reaction for the release of completed polypeptide chains from ribosomes is clearly indicated by the observations that both pea ribosomes (20) and reticulocyte ribosomes (92) will convert amino acids bound to sRNA into "soluble" protein in the presence of an unfractionated cell extract, but not in the presence of the purified "transfer enzyme." The preparation will only transfer amino acids from sRNA to polypeptides bound to the ribosome in the presence of the purified "transfer enzyme." Release of the completed polypeptide chains is obtained only upon the addition of a second protein fraction. The release also requires the presence of ATP and a divalent cation (20, 92, 99, 100). Although the "release enzymes" for both the pea ribosome and the reticulocyte ribosome systems have been purified slightly (20, 92), the mechanism by which the enzyme acts to release polypeptide chains from the ribosome and the manner in which ATP participates in this process are not known. Further insight will probably be gained after the "release enzyme" has been isolated in a pure state.

Secondary changes in the completed polypeptide chain.—Although ribosomes presumably catalyze the formation of single polypeptide chains, many proteins consist of two or more polypeptide chains held together by one or more —S—S— or other covalent cross-linkages. Likewise, single polypeptide chains would appear to be often folded in special configurations (sometimes containing cross-linkages) in biologically active proteins. Although some of these secondary alterations in completed polypeptide chains may be spontaneous, it seems likely that some are produced by specific enzymes. Nothing is known of such reactions or enzymes at present, but the production of completed polypeptide chains by isolated ribosomes should allow appropriate studies to be made.

Structure of the ribosome.—It is not clear as yet whether ribosomes play an active part in the condensation of amino acids to form polypeptide or whether they are passive templates which provide only the information for specifying a particular amino acid sequence. In either event, ribosomes are the key structures in the synthesis of many specific proteins. Their structure is, therefore, of considerable interest, and is being studied by a number of investigators. The general structure of ribosomes from animals, plants, and microorganisms is strikingly similar (101). Likewise, the amino acid composition of ribosomal protein from a number of organisms is markedly similar (102, 103). Ribosomes are spherical structures with diameters of 200 to 300 A. They are composed of 40 to 60 per cent RNA and 40 to 60 per cent protein. The most thoroughly characterized ribosomes are those that have been ob-

tained from *E. coli*, pea seedlings, and rat liver. The spherical ribosome generally observed in extracts of *E. coli* has a sedimentation constant of 70S and a molecular weight of about 2,600,000 (104). The 70S ribosome occurs in the presence of approximately $0.001M$ magnesium ion. If the magnesium ion concentration is increased to $0.01M$, two of the 70S ribosomes aggregate together to form a 100S particle. If the magnesium ion concentration is decreased to approximately $0.00025M$, the 70S ribosome breaks down to one 30S particle and one 50S particle. The 50S particle has a molecular weight of about 1,800,000, and the 30S particle has a molecular weight of about 700,000. All of the particles have about the same ratio of protein to RNA. The 30S particle contains a single molecule of RNA, having a sedimentation constant of 16S and an approximate molecular weight of 560,000 (105). The 50S ribosome is reported to have either one molecule of a 23S RNA (approximate molecular weight of 1,100,000) or two 16S molecules of RNA. X-ray diffraction studies of *E. coli* ribosomes suggest that three-fourths of their

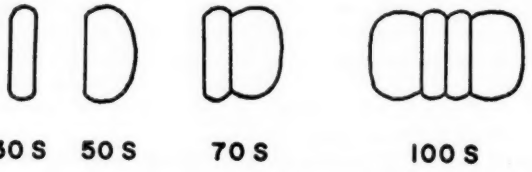

FIG. 2. Sedimentation constants and approximate shapes of the various ribosomal units from *E. coli*.

RNA has a double helical structure (106), but the exact positioning of the RNA is not known as yet. A portion of the RNA appears to be on the surface of the particle and available to the action of ribonuclease (96). However, a considerable amount of RNA is made available to ribonuclease action only if the "normal" ribosome is degraded to the two smaller sub-units by lowering the magnesium concentration (96, 107). This would indicate that a significant amount of ribosomal RNA may be located inside the 70S ribosome. The overall structure of *E. coli* ribosomes has been determined by electron microscopy (108). The relationships of the different structural units of the 30S, 50S, 70S, and 100S particles are shown in Figure 2.

An interesting observation is that disruption of ribosomes to the two smaller sub-units results in the apparent activation of latent enzyme activities. The presence of a latent ribonuclease and deoxyribonuclease associated with ribosomes has been reported (109, 110). Both are activated by disruption of the ribosome at lowered magnesium ion concentrations. There is now reason to believe that these enzymes may be completed molecules that have been synthesized by the ribosome but not released. They are inactive while bound to the ribosome, but become active when they are released by the disruption of the intact ribosome. Siekevitz (111) has reported the release of

synthesized protein from the ribosome by disruption of the ribosome to its sub-units.

The above characteristics of *E. coli* ribosomes appear to be representative of the characteristics of ribosomes in general. The 80S ribosomes of pea seedlings, yeast, and rat liver all exhibit structural characteristics, and sub-units dependent upon magnesium concentration, similar to the *E. coli* ribosomes (101).

It has been observed that the sub-units of pea ribosomes can be recombined by the addition of divalent cations other than magnesium (112). The ability of the ribosomes to incorporate amino acids into protein, however, varies greatly with the cation employed. Only ribosomes formed in the presence of cobalt ions are as active as those formed with magnesium ions. Ribosomes formed with either calcium or manganese ions are essentially inactive. In view of the fact that the sub-units are inactive, and that the divalent cations appear to bind the two sub-units together, it would seem that a critical configuration of the combined sub-units is necessary for protein synthesis to occur. One wonders whether the junction of the sub-units might be the site of polypeptide synthesis.

Investigations on the ultimate protein sub-units composing the ribosomes are still incomplete. Abdul-Nour (96) observed that dodecyl sulfate degraded the proteins of pea seedling ribosomes to uniform 12S sub-units. Yin & Bock (113) found that yeast ribosomes could be degraded even further by treatment with dodecyl sulfate plus urea. The resulting ribosomal protein has an average molecular weight of about 12,000. If this protein is the structural unit of the yeast ribosome, the ribosome would consist of 230 of these sub-units.

The number of kinds of sub-units composing ribosomes has been examined to a limited extent. Yin & Bock (113) found eight different amino acids in N-terminal position in the 12,000 molecular weight sub-units obtained from yeast ribosomes. This suggests at least eight different kinds of polypeptide chains composing these sub-units. In contrast, Tissiéres & Watson (114) found only methionine and alanine end groups in the ribosomal protein of *E. coli*. Likewise, Butler *et al.* (103) found almost 60 per cent of the N-terminal groups of rat liver ribosomes can be accounted for by glycine and proline. These results suggest that ribosomes are not composed of a single kind of polypeptide sub-unit, as appears to be the case with at least some kinds of viruses. Whether these different polypeptides are mixed together in each ribosome, or whether there are different ribosomes with each composed of a single kind of polypeptide sub-unit, is not known.

Mechanism of protein synthesis in other cellular components.—Most of our knowledge concerning the mechanism of protein synthesis is obviously derived from studies with ribosomes. One would like to know whether this is one general mechanism for protein synthesis or whether other cellular proteins are formed by different mechanisms. Unfortunately, our knowledge of protein synthesis by other cellular components is quite limited. There is good

evidence that nuclei, mitochondria, and chloroplasts are independently capable of the synthesis of their particular proteins (3, 5, 9). The synthesis of cytochrome-*c* by mitochondria (22, 23) is an outstanding example of the synthesis of a mitochondrial protein by the mitochondria. Amino acid incorporation into mitochondria has been shown to require a continual supply of ATP, and also to require that fraction of nonparticulate cellular protein which contains amino acid activating enzymes (115). The site of protein synthesis in mitochondria is not yet known. Rendi (116) has reported the presence of ribonucleoprotein particles in mitochondria. These particles become highly labeled during amino acid incorporation by mitochondria. They may act as one site of protein synthesis by mitochondria, and if so, it should be possible to determine whether protein synthesis occurs by the ribosomal mechanism.

Amino acid incorporation into the proteins of isolated thymus nuclei has been studied considerably (117, 127). Evidence that the mechanism of synthesis of at least some nuclear proteins is similar to protein synthesis by ribosomes has been obtained by Frenster *et al.* (118). They were able to isolate ribonucleoprotein particles from lymphocyte nuclei that were active at amino acid incorporation into protein in either the isolated state or in the intact nuclei. Amino acid incorporation required ATP, an ATP-generating system, GTP, and an enzyme fraction from nuclei that contains amino acid activating enzymes. The presence in nuclei of activating enzymes for the various amino acids has been clearly demonstrated by Hopkins (119). The activating enzymes catalyze the synthesis of amino acid-sRNA from amino acids and nuclear sRNA. The sRNA isolated from nuclei contains bound amino acids in the same fashion as cytoplasmic-sRNA. DNA, isolated from nuclei, has no bound amino acids.

Thus, as far as they have been carried, experiments reveal that protein synthesis by mitochondria and nuclei have the same requirements as protein synthesis by ribosomes. The occurrence of amino acid activating enzymes in isolated chloroplasts (120) is also in agreement with this conclusion. Until evidence to the contrary appears, one must regard the ribosomal mechanism as being generally applicable to all cellular protein synthesis.

Additional factors or alternate pathways for protein synthesis.—Although the evidence from a number of laboratories is all in agreement with the general mechanism of protein synthesis discussed above, the possibility of other, as yet unidentified, factors or enzymes participating in the process cannot be excluded until all of the components involved in protein synthesis have been isolated in a pure state and clearly identified. An especially interesting "incorporation factor" has been described by Gale (121). This factor restores the ability of disrupted *S. aureus* cells to incorporate amino acids into protein after this ability has been decreased by the removal of some nucleic acid from the preparation. Evidence that the factor does not necessarily participate directly in protein synthesis has been obtained by Gale *et al.* (122), who find that the factor restores the ability of the preparations not

only to incorporate glycine into protein, but also to incorporate glycine into cell wall material and into RNA. The incorporation factor has been purified by Gale & Folkes (123, 124), and contains glycerol (125). It is probably an unstable derivative of glycerol (126), and the claims that it is entirely glycerol (125) would appear to be premature.

Wagle *et al.* (128) have made the surprising claim that the incorporation factor of *S. aureus* can replace amino acid activating enzymes for amino acid incorporation by rat liver microsomes. This claim is at such variance with the overwhelming mass of evidence for the participation of activating enzymes in protein synthesis, that one must withhold judgement of its significance until it is confirmed by other investigators.

Several investigators have reported results that they believe to be incompatible with the participation of amino acid activation (amino acid-sRNA formation) in the incorporation of amino acids into protein. Thus, Beljanski & Ochoa (129, 130) have described the partial purification of an "incorporation enzyme" which is required for the incorporation of amino acids into the protein of *A. faecalis* particles. This enzyme exhibits no amino acid-dependent exchange of pyrophosphate with ATP, and it is concluded, therefore, that amino acid activation is not involved in the incorporation. It has not been shown, however, that the particles do not contain bound amino acid activating enzymes, that the incorporation enzyme might not be an enzyme capable of transfer of amino acids from sRNA to protein, or that the incorporation enzyme might be capable of the synthesis of amino acid-sRNA without exhibiting a pyrophosphate-ATP exchange. The claim that the purified "incorporation enzyme" can replace amino acid-activating enzymes for amino acid incorporation by rat liver microsomes must be regarded with caution, especially since it has not been possible to confirm it in other laboratories (131).

Thus, as stated at the beginning of this discussion, a picture is emerging of the chemical steps involved in protein synthesis. We can confidently expect that the next few years will witness the elucidation of the details.

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PHOTOSYNTHESIS UNDER NATURAL CONDITIONS[1]

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Introduction

Of all aspects of plant metabolism, photosynthesis shows the most prominent variation under the dictates of the immediate environment. This sensitivity, and the dominance of the process in the synthetic economy of nature, has stimulated its study under more or less natural conditions during the past forty years. Nevertheless, a majority of physiologists have regarded with disfavour the unstandardised and arbitrary limitations of "natural conditions"; such experimental conditions are generally not easily attained, even in field experiments. There appears to be no comprehensive and coherent organisation of the data now available. The principal difficulties lie in the incomplete integration of laboratory and field observations, work on terrestrial and aquatic vegetation, and measurements of photosynthesis and growth yields.

Studies on land plants developed rapidly in 1918 and the years immediately following, particularly those by Boysen-Jensen (19), Henrici (79), Lundegårdh (118, 119), and Stålfelt (195). They benefited especially from the kinetic foundations laid by Blackman and his school in earlier decades (e.g., 5), and by Willstätter & Stoll (257) in 1918. The unique value of aquatic plants, particularly unicellular algae, was less readily appreciated for illustrating photosynthetic processes in nature than for the laboratory work on the photosynthetic mechanism that followed the pioneer experiments of Warburg. The use of these organisms developed from field experiments by marine and freshwater biologists in the 1920's [e.g., Gaarder & Gran (53), Gail (58), Marshall & Orr (125) and Ruttner (173, 174)], and has recently shown a spectacular expansion in connection with measurements of the photosynthetic productivity of plankton [see reviews in (66, 206, 243)]. General discussions of the photosynthesis of terrestrial plants in nature are given by Boysen-Jensen (20), Lundegårdh (122), Rabinowitch (168), Sauberer & Härtel (184), Walter (248), Wassink (250), and Zalensky (264); reviews in the *Encyclopaedia of Plant Physiology* (1960) appeared too late to be considered here. A more restricted but active field of study, the photosynthesis of trees, is reviewed by Kramer (107) and by Kramer & Kozlowski (109).

The peculiar problems presented by photosynthesis in nature chiefly concern the kinetics of the process. The photosynthetic rates in any vegetation cover are controlled by a considerable number of internal and external factors and by interactions between these factors [see Thomas (226)], and appar-

[1] The survey of literature pertaining to this review was concluded in July 1960.

ently offer little prospect for a unified treatment. However, light, as the energy source, can be regarded as the master factor, and shows more remarkable variation than any other environmental factor. Much behaviour can be interpreted in relation to the rate-intensity characteristic of photosynthesis, its modification in response to other factors, and its expression in the variation in space and time of photosynthetic rates under natural conditions. This characteristic also expresses the two universal limitations of the efficiency of energy utilisation: the maximum quantum yield at low intensities, and light-saturation at high intensities. It consequently provides a starting-point for comparing observed with potential yields.

The conversion of net photosynthesis to gross photosynthesis by correcting for respiration losses is often uncertain, partly because of possible changes in respiration during illumination (e.g., 38, 110, 197); therefore, many field measurements of photosynthesis are expressed as net rates only. Where possible, however, this discussion will be based upon the probable gross photosynthesis, with the consequences of respiration introduced separately.

Sources of Information

In most field work small samples of plant material have been used, exposed in some form of transparent chamber, cuvette, or bottle. Here the photosynthetic uptake of carbon dioxide or liberation of oxygen could be measured either directly or following the absorption of residual carbon dioxide from an air-stream in alkali solution. Changes due to carbon dioxide uptake by both terrestrial and aquatic plants have been estimated by differences in titration, electrical conductivity, pH, and—in recent years—by fixation of radiocarbon (^{14}C). Infrared absorption analysis, which enables the sensitive and continuous recording of carbon dioxide in air, has been much exploited recently in work on land plants (e.g., 10, 12, 88, 161, 163). The evolution of oxygen is readily measured only with aquatic plants, for which the Winkler method has been widely used. A brief survey of methods employed for terrestrial plants is given by Frenzel (52), and for aquatic plants by Lund & Talling (117). Work with entire terrestrial plants or stands usually requires larger and more elaborate plant chambers; examples are described by Boysen-Jensen (20), Heinicke & Childers (77), and Thomas & Hill (227, 228). Any enclosure involves a danger of the development of special conditions of the *Küvettenklima*, particularly by overheating in air (cf. 5, 17, 73, 229) and reduced mixing in water (cf. 59, 61). Various tests of these effects, and some suggested remedies such as improved circulation or cooling jackets, have been described [e.g., Bosian (18), Koch (102), Talling (220), and Tranquillini (229)]. These difficulties are absent, but others—especially those attributable to translocation—are present in work based on the measurement of assimilates formed by leaves (68, 69, 101, 143, 181, 186, 225).

Field results generally fall in two main groups that are governed by the differing opportunities for exposures between terrestrial and aquatic vegetation. With the former, the diurnal variation of photosynthetic rate has been studied intensively by successions of short exposures, but the vertical varia-

tion of rates in the leaf canopy is rarely investigated. For aquatic plants the variation of rate with depth has been the centre of study, with diurnal changes often obscured in long exposures. Besides these complementary deficiencies, the data often permit only qualitative, or semi-quantitative correlations to be made between the variation of photosynthetic rate and the more important environmental variables such as light intensity, temperature, and carbon dioxide supply.

Advantages offered by a recourse to laboratory exposures are here obvious. In the first instance, such experiments allow the comparison of some photosynthetic indices using plant samples collected in different situations. Examples are provided by studies of the variation of photosynthetic capacity with latitude (e.g., 144, 212) or season (12, 64, 127, 162, 182, 257), and of differentiation between "sun" and "shade" material (10, 20, 207). The application of such measurements to a reconstruction of photosynthetic activity in nature requires more extensive sampling and measurement of environmental variables than is usually made; direct comparisons of laboratory and field exposures (e.g., 220) are rarely attempted. This "synthetic" approach to the estimation of photosynthetic activity under natural conditions was most strikingly developed by Boysen-Jensen and his associates (20, 21, 22, 113, 172). Their contributions are among the most stimulating to the subject, although some oversimplification was inevitable and was criticized by others [e.g., Filzer (48), Guttenberg & Buhr (69)].

Some attempts have been made to avoid the limitations of small enclosed samples by estimating the total photosynthesis of a plant community from the diurnal changes produced in the gaseous composition of the ambient medium. For small water bodies the changes are often confined in horizontal and vertical directions and are relatively accessible for study. Measurements for a representative water column of dissolved oxygen [see Juday *et al.* (95), Talling (219), Vinberg (242, 243)] and carbon dioxide [deduced from pH data, e.g., Park *et al.* (157), Verduin (239, 240)] have been used; the chief uncertainties arise from the variable exchange between water and atmosphere. The diurnal changes of atmospheric composition produced by land vegetation are more susceptible to turbulent mixing and dissipation, but in favourable weather conditions can follow a regular course and affect an air-column of considerable height [e.g., 100 m.—Chapman *et al.* (31), Huber (85, 86)]. Their use in estimating photosynthetic fixation of carbon dioxide is discussed by Huber (84 to 87).

Lastly, the carbon increase indicated by plant growth can be regarded as an integration of net photosynthesis with time. However, there are few quantitative comparisons with gasometric data, although such are invited by one particularly successful technique of growth analysis.

Potential and Actual Yields

The simplest model of a photosynthetic cover of vegetation would be provided by an uniform algal suspension, absorbing all incident light and operating at the maximum quantum yield of photosynthesis. In illustration

one may assume an average of 0.1 molecules oxygen per quantum for the latter [Rabinowitch (168)], corresponding to an average efficiency of solar energy utilisation in the spectral region of 400 to 700 mμ, of about 22 per cent (e.g., 54, 252). The photosynthetically available energy in this spectral region normally accounts for a fraction, close to 0.46, of the total energy received (e.g., 43, 54, 217, 250). Consequently, a radiation total of 600 cal./cm.^{2} (horizontal surface), common on very sunny days at many latitudes (cf. 184, 192), would lead to a photosynthetic production of carbohydrate that was equivalent to 65 g. C/m.^{2} per day. This value is a measure of the gross activity of an ideally efficient vegetation cover under conditions of radiation a little below the maximum experienced on the earth. With it, the yields of less efficient models of vegetation, as well as yields actually observed, can be usefully compared [cf. Fogg (49)].

Two other model situations, readily visualised, may be founded on the experience of photosynthetic rates measured per unit leaf area. At lower light intensities, in which the rates increase linearly with increasing intensity, increases of 1.5 to 2.0 mg. CO_2/100 cm.^{2} per hr. in 1 kilolux increments of intensity are often observed. If a daily energy total of 600 cal./cm.^{2} [approximately 770 kilolux-hours (192)] were entirely utilised in this way, in a completely absorbing leaf cover, a fixation of 31 to 42 g. C/m.^{2} per day would result. This figure is again an upper limit of yield, as light-saturation of photosynthesis is likely to be reached during a considerable part of the supposed sunny day. One method for estimating its effects is proposed by de Wit (260). Photosynthetic rates of healthy leaves at light-saturation commonly lie between 10 and 25 mg. CO_2/100 cm.^{2} per hr. in air not enriched with CO_2 (168, 212). If these persist during a 12-hour day with a uniform and uninterrupted vegetation cover that is one leaf in thickness, the corresponding fixation will be 3.3 to 8.2 g. C/m.^{2} per day.

Values of the actual yields of vegetation can be based upon measurements for short periods (e.g., one or several days) or much longer times (e.g., a growing season, or a year). It was noticed long ago in Europe [e.g., Mayer (126)] that the annual dry weight yields of many dense tree and crop stands were of remarkably similar magnitude, usually 5000 to 10,000 kg./hectare. Later experience (cf. 21, 25, 134, 136, 137, 156, 158, 159, 248, 250) has generally confirmed this magnitude, but has also indicated some appreciably higher yields (15,000 to 35,000 kg./hectare). If the total dry weight contains an average carbon content of 40 per cent (values commonly lie between 30 and 50 per cent), the higher range quoted above corresponds to average net fixation rates of 1.6 to 3.8 g. C/m.^{2} per day in a maximum growing season of 365 days. Owing to respiration and other losses, the corresponding gross photosynthesis is often likely to be 50 to 100 per cent higher [e.g., Heinicke and Childers (77), Möller *et al.* (135, 136)]. The gross photosynthetic yields of dense natural populations of planktonic algae, measured by gas exchange during short periods, reach values of 3 to 4 g. C/m.^{2} per day (e.g., 171, 201, 206, 208, 219).

These yields are clearly far below those permitted by the maximum quantum yield of photosynthesis. The gap is equally evident in numerous other comparisons based upon percentage energy conversion over long periods [e.g. Gaastra (54), Rabinowitch (168), Wassink (250, 251)]. However, recent work with both algae and terrestrial plants has shown that, in short periods under favourable field conditions, the gap between actual and maximum potential yields can be considerably reduced. Improved growth yields in algal mass cultures under natural illumination can represent an overall energy conversion (spectral region 400 to 700 mμ) of around 6 per cent and a net carbon fixation of approximately 5 to 10 g./m.^{2} per day [Oorschot (155), Tamiya (222)]; and the maximum daily yields of some crops [see Blackman & Black (7), Gaastra (54), Kamel (97, 98), Wassink (250)] are of similar magnitude.

The preceding estimates of yields in model situations suggest that much of the difference between actual and potential values originates from light-saturation of photosynthesis under natural conditions. It is also indicated by several analyses of actual yields (e.g., 54, 147, 218, 252). More specific expressions of this effect are discussed below.

The Expression of Light-Saturation under Natural Conditions

Rate-intensity curves of photosynthesis almost universally show a transition between a linear relation of rate and light intensity when the latter is low, and a maximum rate which usually persists over a considerable range of higher intensities. This transition to light-saturation is often gradual, especially in optically dense material [e.g., Boysen-Jensen & Müller (24), Rabinowitch (168)] but can be most precisely indicated by the intensity (I_k) at which a continuation of the initial gradient (θ) would reach the light-saturated rate (P_{max}) [e.g., Talling (217)]. The more common visual estimates of the intensity at which light-saturation occurs are generally about twice this I_k value. The I_k index also has the advantage of linking P_{max} and θ characteristics by the simple relation $P_{max}=\theta \cdot I_k$, and can be reinterpreted in terms of other mathematical formulations of the rate-intensity characteristic (e.g. 217). A division of I_k by the ratio between P_{max} and respiration rate (R) (i.e., P_{max}/R, related to the "economic coefficient" and "balance quotient" of many European authors), yields the value of the compensation intensity if this falls within the initial linear region of the rate-intensity characteristic (217).

The variation of I_k can be deduced from its two determinants, P_{max} and θ. The former is the most variable, and so accounts for most of the changes in I_k. Illustrative examples are provided by the sensitivity of P_{max} and I_k to temperature [e.g., Müller (144); Talling (217); Ruttner (173)] and stomatal aperture [Stålfelt (196)], the depression of P_{max} being associated with reduced I_k values. It is therefore dangerous to interpret in isolation the variable onset of light-saturation, as is often done in the description of supposed heliophilic or ombrophilic characteristics. Thus Boysen-Jensen (20) and Boysen-Jensen

and Müller (24) pointed out the frequent similarity of the initial gradient (θ) in sun and shade examples of different species and in leaves of the same species; here saturation at low light intensities in the shade forms was largely imposed by correspondingly low rates at light-saturation, assessed per unit area of leaf or thallus.

Measurements of photosynthetic rates in the field express the consequences of light-saturation in several ways. It is usually evident in diurnal curves of photosynthesis based upon small plant samples [e.g. (125, 164); cf. however, Cartellieri (29)] and in rate-intensity curves calculated therefrom (30, 100, 161, 230, 241). Another illustration is given by comparative measurements of photosynthesis below artificial or natural light screens of different densities [e.g., Lundegårdh (118), Ryther (175), Stålfelt (195)]. A pronounced light-limitation of photosynthesis is often shown only with daylight factors (the fractional or percentage illumination relative to full daylight) of <30 per cent; such factors are often called "light intensities," but represent much more complex light conditions in varying natural illumination [see Bormann (15)]. In the most interesting situation of this kind, best illustrated by work with planktonic algae, the light screen is provided by varying depths in the plant community under study. Light-saturation can then be shown in a depth zone with maximum photosynthetic rates, passing below into a near-exponential decline of rates with increasing depth, and often ending above in a superficial zone with depressed rates associated with strong illumination [e.g., Jenkin (93), Manning & Juday (123), Steemann Nielsen (204), Talling (218, 219)].

Such depth-profiles can be related to basic photosynthetic characteristics by analysis, and to the overall rate under unit area of cover by integration. Thus they afford a means of connecting the integrated yields per unit area with basic photosynthetic characteristics. The maximum rates possible in these profiles, some special problems linked with their upper and lower regions, the thickness or density of the plant cover through which they extend, their integration in relation to depth and time, and comparison with an alternative approach of growth analysis are discussed below.

Variation of Photosynthetic Rates at Light-Saturation

Photosynthetic rates measured at (or near) light-saturation can be used to compare the absolute capacities of plant material differing in type and origin, and to illustrate the effects of factors with ecological significance. Compilations of such rates for various terrestrial and aquatic plants are given by Rabinowitch, Table 28. VI in (168), Stocker (210, 212), and Verduin (238).

Ecological consequences can be anticipated for the well-known increase of light-saturated rates with temperature, depression and inhibition at very high and low temperatures, sensitivity to previous thermal history [e.g., Harder (71)], and the effects of the temperature-dependence of respiration rate upon net photosynthesis. The abundant records of daily photosynthesis

by land plants seldom indicate any strong positive influence of diurnal temperature change (e.g. 29, 30, 80, 228, 241). Demonstration of such influence is also hindered by the problems of measuring true leaf temperatures and allowing for over-heating effects in small chambers. These difficulties are much reduced with water plants, for which Gessner (60) describes some probable effects of diurnal temperature change. Diurnal changes of temperature are usually much greater in terrestrial than aquatic environments. In the former, high leaf temperatures, especially in the afternoon, are often accompanied by depressed rates of photosynthesis [e.g., Harder *et al.* (73)]. Besides the direct depression of photosynthetic rates at high temperatures [Stålfelt (198, 199)], several indirect consequences of temperature, affecting water deficit and respiration rate, can be involved.

Some pronounced examples of the diurnal or seasonal depression of net photosynthesis, which could be related to the rise of respiration rate in warmer conditions, are recorded for desert plants [Harder *et al.* (73)], for various mosses and lichens [Romose (172), cf. Stålfelt (197)], and for conifers [Pisek & Winkler (162), Tranquillini (230)]. In the algae, laboratory studies of some thermophilic strains of *Anacystis* and *Chlorella* show the utilization of high temperatures for achieving remarkably large rates of photosynthesis and growth [see Sorokin (193)] and noticeably high rates may also be found in tropical freshwater plankton under field conditions [Prowse & Talling (167), Talling (219)]. The many rates recorded for phytoplankton from other regions, assessed per unit chlorophyll content, are usually in the range of 3 to 15 mg. O_2/mg. hr. (or its equivalent) at light-saturation (e.g., 62, 63, 64, 90, 123, 262). Tropical examples of terrestrial plants studied do not appear to show particularly high photosynthetic rates at light-saturation [Stocker (211, 212), Walter (246, 248), Zalensky (264), cf. also (34, 74)].

The effects of low temperatures upon the photosynthetic activity of land plants in nature have been much investigated, particularly in relation to the performance of evergreen plants in winter [e.g., Cartellieri (28, 29), Freeland (50), Pisek & Rehner (160), Pisek & Tranquillini (161), Tranquillini (231)]. There is general agreement that significant, if reduced, photosynthesis can occur at air temperatures below 0°C. Tranquillini (232) describes development of the photosynthetic capacity of leaves of *Pinus cembra* still covered in alpine snow.

The limitation of photosynthetic rates in nature by the carbon dioxide supply is generally agreed to be most pronounced in terrestrial habitats, where the atmospheric concentration of carbon dioxide is low and averages about 0.03 per cent or 0.59 mg./l. near sea level. Deviations from the mean have been studied extensively (see 89, 120). The physiological significance of the most regular form of variation, the decrease with altitude, is little understood [cf. Decker (39), Verduin (238)]. Despite the low absolute—though not relative—carbon dioxide concentrations at high altitudes, there are recurrent reports of unusually high photosynthetic rates by alpine plants in nature [Blagowestschenski (9), Cartellieri (29), Zalensky (264)]. Daily changes of

concentration are also very common in and near vegetation (e.g., 30, 85, 86, 212, 224) and often are measured in records of the diurnal course of photosynthesis. However, a pronounced correlation between concentrations and rates is rarely evident [see, however, Blagowestschenski (9), Kaben (96), Mönch (138), Uhl (235), Wilson (258)] and the total daily variation of concentration is usually not large, though an air column of considerable thickness may be involved. High concentrations under a canopy near the soil have been supposed to offset the less favourable light conditions there [e.g., Kaben (96), Lundegårdh (118, 119, 120)], but direct proof is difficult and in some cases the evolution and accumulation of carbon dioxide was probably overestimated [cf. Evans (44), Stocker (212), for tropical forest conditions]. Wind movements have an obvious importance for such distribution patterns, and there are some quantitative data for the effects of low velocities on the supply of carbon dioxide to photosynthesizing surfaces [e.g., Denecke (40), Wadsworth (245), Walter & Zimmermann (249)]. The stomatal control of carbon dioxide supply has been much discussed in relation to midday or afternoon depression of photosynthesis (see below). Variation in stomatal aperture probably also accounts for some differences in the photosynthetic activity of leaves at different levels in vegetation [e.g., Lundegårdh, Fig. 51 in (121), Pisek & Tranquillini (161)], and possibly for some seasonal differences [Cartellieri (28)].

There are fewer data for aquatic plants on the relation between carbon dioxide supply and photosynthetic rates in nature. The limitations of diffusion to massive plant organs in water are well known [e.g., Gessner (59, 61), Hammann (70)], so that water currents and turbulence are likely to be of considerable ecological significance. Such indications are generally lacking for the dilute natural suspensions of planktonic algae [e.g., Talling (220)], with a much greater surface to volume ratio. The photosynthetic removal of carbon dioxide from the natural carbonate-bicarbonate buffer system is reflected in a rise in pH. Many diurnal examples have been described [e.g. (157, 219, 240)], and some were used for the estimation of daily photosynthetic productivity. However, a severe limitation of photosynthesis by carbon dioxide depletion is only likely at pH values higher than are commonly found. A probable example for freshwater plankton algae is described by Steemann Nielsen (202). Some species, such as *Arthrospira platensis* [Jenkin (92)], show a remarkably dense development and active photosynthesis in very alkaline waters with pH exceeding 10.5. There is a considerable amount of evidence for the utilization of bicarbonate as carbon dioxide source by many aquatic plants [see Gessner (66)].

Seasonal changes of photosynthetic capacity, which reflect the previous history of the plant material, have been described from both field exposures and from standardised laboratory measurements combined with periodic sampling of vegetation. The first frosts of winter lead to a sharp fall in the maximum photosynthetic rates of many land plants [Bordeau (12), Pisek & Tranquillini (161), Pisek & Winkler (162), Tranquillini (231)]. In evergreens

relatively low photosynthetic capacities are usually found in the winter period (12, 28, 127, 161, 182), although recovery may occur in a few days in warmer conditions [Bordeau (12), Pisek & Winkler (162)]. The variation of photosynthetic activity with age of individual leaves [e.g., Freeland (51), Richardson (169), Stålfelt (195)], is also a source of seasonal variation. Seasonal rains in hot dry regions can produce a remarkable rise in photosynthetic rates [Guttenberg & Buhr (69), Harder *et al.* (73), Stocker (209, 213)], although—as in tidally exposed seaweeds [Stocker & Holdheide (214)]—the dry phase need not represent a "resting season" for photosynthesis (73).

A diurnal depression of photosynthetic capacity has been very commonly found for land plants [Rabinowitch, pp. 873–76 in (168)], and is also recorded for aquatic macrophytes [Meyer (129)]. In recent years examples have been described for marine and freshwater phytoplankton (41, 83, 153, 154, 178, 189), with maximum capacities usually reported near dawn and minimum near sunset. This daily decline may be connected with the photo-inhibition often found in short exposures near the water surface; Shimada (189) gives evidence for parallel changes in chlorophyll-*a* content. Behaviour in terrestrial plants is more varied, often with slight to large depressions near midday, persisting in some examples throughout the afternoon. The occurrence of these patterns in the same species at different altitudes is described by Zalensky (264). No single explanation can fit all the examples described; there is evidence for the effects of stomatal control [e.g., Nutman (150, 151)], the direct inhibition of photosynthesis at high temperatures and light intensities [cf. Stålfelt (198, 199)], accumulation of assimilates [e.g., Guttenberg & Buhr (69), Kurzanov (111)], water deficit [e.g., Bosian (17), Kjaer (101), Stocker (213)], and—for net assimilation—increased respiration rates at higher temperatures [Harder *et al.* (73), Polster (164), Tranquillini (229)]. As almost all the data are derived from diurnal exposures in natural illumination, it is difficult to separate the effects of depressed rates at light-saturation (attributable to the previous history of the material) from the unfavourable influence of the immediate environment [see, however, Kjaer (101)]. Kostychev and his co-workers (e.g., 103) stressed the importance of internal factors in determining large diurnal fluctuations of photosynthetic rate, but these views were not supported by much other work [(11, 23, 42, 168); cf., however, Zalensky (264)].

Several comparisons have been made of the maximum photosynthetic rates shown by plants from various terrestrial habitats or climatic zones [Rabinowitch, Table 28.VI in (168), Stocker (212), Walter (246, 248), Zalensky (264)]. The first field survey of this type on a large scale was undertaken by Kostychev and his associates (104, 105, 106). The high rates described for various alpine plants and the comparatively modest rates for some tropical plants have already been mentioned. It appears that high rates of photosynthesis are often achieved in apparently unfavourable habitats with a short growing season the brevity of which is thereby partly compensated; there are examples of this for alpine [Cartellieri (29), Zalensky (264)], arctic

Müller (144)], and desert [Harder *et al.* (73)] plants. Continuous photosynthesis under arctic summer conditions is described by Kostychev *et al.* (106) and Kisliakova (100); cf. also Müller (144). Zalensky (264, 265) considered that the variation of photosynthetic capacities of species in continental climates, as in Central Asia, was greater than in the floras of more oceanic or tropical climates. Succulent halophytes do not appear to show marked peculiarities of gross photosynthetic behaviour, although rates expressed on a fresh weight basis are naturally low and the ratio of rates of respiration to photosynthesis on a unit area basis may be unusually high [Beiler (1), Gabrielsen & Larsen (57), Neuwohner (148)]. Work on one halophyte, *Aster tripolium* (57), provides one of the few clear examples of different photosynthetic responses between ecologically distinct forms of the same species (see also 2, 46, 88, 133).

Some Limitations of Photosynthesis in Exposed and Shaded Situations

These limitations are shown most diagrammatically in many of the depth-profiles of photosynthetic activity reported for planktonic algae, and have been noted briefly. The depression of photosynthetic rates near the water surface is common at light intensities exceeding 100 kiloerg (400 to 700 mμ)/cm.^{2} per sec. [Edmondson (43), Jenkin (93), Manning & Juday (123), Manning *et al.* (124), Talling (217, 219, 220)], and a depth corresponding to 1 per cent of the surface intensity is usually taken as the lower limit of the zone in which appreciable photosynthesis can occur. The overall significance of the surface depression is very uncertain in relation to algal populations circulating over a greater depth range; it may affect subsequent rates of photosynthesis at light-saturation. There is also some evidence for a damaging influence of radiation, such as the ultra-violet, rapidly absorbed by the water (67, 124, 128). The reduced photosynthesis often shown near the water surface, when the algae were sampled from the depths at which they were later exposed may often be caused by decreased population density rather than specific rates of photosynthesis (cf. 205). The decline of photosynthetic rate at greater depths reflects the exponential decline of light intensity. It bears no constant relation to the relative or percentage light values, but is also determined by the logarithmic interval between the surface light intensity (I_o) and the intensity that measures the onset of light-saturation (I_k). The greater this interval, the deeper the depth-profiles are likely to extend [Talling (218)]. This source of variation is dwarfed, however, by the enormous range of light penetration in natural waters (32, 185) which leads to a variation of photosynthetic zones from 100 m. or more in the clearer oceans [Steemann Nielsen & Jensen (208)] to 1 m. or less in highly coloured and turbid waters [e.g., Curtis & Juday (36), Manning *et al.* (124), Prowse & Talling (167), Talling (219)].

The comparative photosynthetic rates of aquatic macrophytes at differ-

ent depths have been studied less, but similar restrictions at shallow and great depths are known in fresh waters [Hogetsu (81), Meyer & Heritage (130), Meyer *et al.* (131), Potapov (165), Ruttner (173, 174), Yoshimura (263)] and in the sea [Gail (58), Levring (115), Montfort (140, 141, 142), Printz (166), Tschudy (234)]. Attached algae and mosses have been found growing at depths which probably correspond to about 1 per cent or less of the effective surface radiation [e.g., Juday (94), Oberdorfer (152)], although few adequate measurements of light penetration are available. The significance here of adaptation to the colour or intensity of light has been much discussed [see (65, 115, 142, 152, 185, 187)].

The light field is usually more heterogeneous in a terrestrial habitat [see Sauberer & Härtel (184)] than under water, and the consequences of a vertical gradient for photosynthesis are less easily demonstrated. Measurements of such gradients are discussed by Sauberer & Härtel (184) and Monsi & Saeki (139), but most light data apply to a single stratum in the plant cover, such as a forest floor. As under water, the 1 per cent light level often marks the lower limit of appreciable photosynthetic activity, and values of this order are common at the soil surface beneath dense vegetation. This light level is also likely to represent the region of the compensation point during short exposures, as intensities of 20 to 80 kilolux are typical of average daily illumination with 0.2 to 0.8 kilolux for the compensation intensity. Some extreme shade plants can grow at lower relative intensities, some measured as below 0.1 per cent. Biebl (4) describes a particularly interesting example for the moss *Mnium serratum* inhabiting rock-cavities, where the deepest shade tolerated was given as 0.024 per cent, with a midday maximum of light intensity at about 26 lux. On exposure to full sunlight, death occurred within a few hours. Under a vegetation cover the assessment of the effective light climate is rendered more difficult by spectral modifications due to the leaf canopy (33, 188), the short-period fluctuations due to sun-flecks (45, 119), and long-period changes connected with leaf development and fall [e.g. (184), Fig. 48]. Daxer (37) [cf. also Kaben (96)] studied in detail the depression of photosynthetic activity that followed development of the tree canopy. Some species, such as *Anemone nemorosa* [see also Löhr (116)], showed a change from a positive to a persistent negative balance of net photosynthesis, and in others (e.g., beech seedlings, *Dryopteris filix mas*) the positive balance was regained after a shift in the compensation point.

Various factors that can reduce the light-saturated photosynthetic capacity are often most pronounced at the upper exposed level of a vegetation cover. A detailed exploration of the distribution of photosynthetic activity in the tree-crowns of *Picea excelsa* and *Fagus sylvatica* was made by Pisek & Tranquillini (161). Here reduced photosynthesis could be found associated with water deficit and stomatal closure in exposed shoots subjected to greater insolation. Photosynthetic activity extended most deeply on the more illuminated side, particularly at low solar elevations. Earlier, Filzer

(47) showed differences of photosynthesis between north and south aspects of trees that were connected particularly with light intensity, and others between east and west aspects with diurnal timing. The significance to photosynthesis of the angular distribution of light has been little studied in either aquatic or terrestrial habitats, although the ecological application of photometers recording spherical illumination have been described for both habitat types (e.g., 132, 149). Stocker (212) has emphasized the probable importance for photosynthesis of the diffused reflection by leaves in some tropical vegetation.

The differentiation of photosynthetic and other characteristics between plant individuals, or their parts, in exposed and shaded situations is very common, but there is less agreement on its adaptive significance. "Shade plants" and "shade leaves" are discussed by Boysen-Jensen (20) and Lundegårdh (121, 122). They often show relatively low rates of respiration and maximum photosynthesis, with light-saturation and the compensation point found at low intensities. The last two features have elicited the most comment, but can usually be interpreted in terms of the first two in conjunction with a less variable initial gradient of the photosynthesis-intensity curve. Examples of woodland plants have been particularly studied in this connection [Boysen-Jensen (19), Löhr (116), Lundegårdh (118, 122)]. The differentiation between sun and shade leaves on the same plant [e.g., Kusomoto (112), Pisek & Tranquillini (161), Tranquillini (230)] is most fully described for the beech, *Fagus sylvatica* [Boysen Jensen (20), Müller (146), Pisek & Tranquillini (161)]. Differentiation between species was well illustrated in a survey of eight "sun" and five "shade" plants by Böhning & Burnside (10), although the authors emphasized that the ecological significance is not simple, except in habitats with very low light intensities. The differentiation of "sun" and "shade" characteristics of photosynthesis has been described recently in relation to depth in phytoplankton communities [Ryther & Menzel (177), Steemann Nielsen & Hansen (207)]; the samples of deeper origin showed light saturation at lower intensities and a greater susceptibility to photo-inhibition at higher intensities.

Ontogenetic adaptation or lability [Harder (72)] in relation to light intensity, leading to a differentiation of "sun" and "shade" characteristics, is known for both algae and for higher terrestrial plants. These modifications were studied by Wassink *et al.* (253) in leaves of *Acer pseudoplatanus* that were exposed to various intensities of artificial illumination and fractions of full natural illumination. Changes in the two series were qualitatively similar, with an increase in the light-saturated rate of photosynthesis and the onset of light-saturation with rising light intensity during adaptation, and a corresponding decrease in the chlorophyll content per unit area. Ontogenetic differentiation of photosynthetic characteristics has also been described by Burnside & Böhning (27) and by Bordeau & Laverick (13) for several species of conifer seedlings. The latter emphasize that exceptions to the typical differentiation of "sun" and "shade" behaviour occur. Shade leaves

usually show a high chlorophyll content per unit weight, though—owing to their frequent thinness—not always per unit area.

The Density of Photosynthetic Cover in Nature

The density of photosynthetic material in the unit area of a natural habitat is an essential link between data on the photosynthetic behaviour of plant samples and the overall photosynthesis of the plant cover. However, systematic and comparative measurements in relation to photosynthesis under natural conditions are comparatively few.

In some respects the simplest situation is found in natural populations of planktonic algae, whose chlorophyll content (particularly chlorophyll-*a*) has been much studied in recent years. Most measurements relate to the content per unit volume of water; and although any limits are arbitrary, values of <1 mg./m.^{3} are common in the unproductive open oceans (e.g., 35, 82), 1 to 30 mg./m.^{3} in moderately productive waters (e.g., 179), and >30 mg./m.^{3} in very productive regions (e.g., 63, 180). A few calculations have been made of the content below unit area in a complete water column. Gessner (64) estimated that in productive waters the chlorophyll content could reach values of about 1 g./m.^{2}, similar to those found in closed terrestrial communities. The more relevant content, however, is that of the illuminated part of the water column (euphotic zone) in which appreciable photosynthesis is possible. The maximum content will depend on the extinction associated with unit density of the algal suspension. Steemann Nielsen (203) used absorption data of chlorophyll extracts in organic solvents to estimate roughly the maximum content as about 300 mg./m^2., which appeared compatible with actual amounts per unit area in dense natural and cultured populations. It is also supported by observations on the increase of the light extinction during the growth of a planktonic diatom in nature [Talling (221)]. The increment of optical density ($\ln I_o/I$) associated with unit density of algae then appeared low enough (of the order of 0.02 per mg. chlorophyll-*a*/m.^{2}) to render unlikely any strong optical incursion by the densities of phytoplankton most commonly found in nature.

In most terrestrial vegetation the situation is obviously very different, both as regards the optical importance and the density per unit area of photosynthetic material, and its usual display in flat laminae. Steemann Nielsen (203) considers that the density of chlorophyll often reached per unit of ground area is significantly higher than the maximum value possible for phytoplankton in the euphotic zone. He connects this difference with the optical advantages offered by leaf arrangement and structure, particularly the combination of diffusing epidermis and light channels in palisade cells. The predominant laminar arrangement allows the density of photosynthetic cover to be conveniently expressed by a pure number, the ratio of leaf area to ground area. In recent years this measure—the leaf area index—has been widely used in analyses of crop growth (7, 54, 97, 145, 254), light interception by vegetation (26, 139, 149), comparisons of plant cover (236), and in

theoretical treatments of photosynthetic productivity (91, 99, 139). It was also involved in earlier Scandinavian work on terrestrial "Stoffproduktion" (e.g., 20, 22, 114). Watson (254) gives an excellent general review in relation to crop plants, and some ecological aspects are treated by Walter (248).

In continuous plant cover almost all values of leaf area index fall between one and eight; low values of about one are probably common in shade habitats [cf. Lundegårdh (118)], and higher values of up to eight are illustrated by some European beech woods [Möller (134)]. The upper limit of the index is clearly connected with the effective light extinction associated with each leaf layer. The relationship between leaf area index and vertical light extinction, indicated by Brougham (26), Monsi & Saeki (139), Nichiporovich & Chmora (149), appears to be less strongly influenced by the average transmission (usually near 10 per cent) of individual leaves than by the type of leaf arrangement [for which see Boysen-Jensen (20)]. With the very thin leaves of mosses the leaf area index can be correspondingly high [Romose (172)]. Light extinction is likely to be more directly related to leaf area index than to chlorophyll content per unit area, since much variation in the chlorophyll content per unit area of leaves (especially >5 mg./dm.^{2}) has a small influence on either optical density [e.g., Kasanaga & Monsi (99), Shulgin & Kleshnin (191) or photosynthetic efficiency in weak light (Gabrielsen (56)].

Depth-Integration of Photosynthetic Rates

The most complete assessment of the total photosynthetic activity below unit area of a plant cover would be provided by the summation of individual rates recorded at representative points within the cover. This approach has been applied extensively using the relatively homogenous communities of planktonic algae. Here the area enclosed by the depth profile of photosynthesis represents the required depth-integral of photosynthetic rate. It is susceptible to the depth-limitations seen in exposures of uniform samples and also to the effects of any stratification that affects population density, composition, and differentiation of photosynthetic characteristics, between cells at various levels. The unravelling of these simultaneous variables has rarely been attempted. The effects of population density can be seen in the replotting of depth profiles with specific photosynthetic rates per unit quantity of pigments [Currie (35)], or in comparisons with crop profiles [Steemann Nielsen (205)]. The separation of depth changes as due to the vertical light gradient, from the differing photosynthetic capacity of population samples from various depths, has been used in several indirect methods of estimating photosynthesis per unit area. Photosynthetic capacity has been measured for samples exposed in illuminated constant temperature baths [see Steemann Nielsen (200, 201, 206)], or near the water surface with natural illumination [Sorokin (194)]. Little is known of the quantitative influence of a depth-differentiation of "sun" and "shade" characteristics upon the depth-integral of photosynthesis.

The influence and interaction of individual variables also can be tested by a mathematical integration of photosynthetic rates, aimed at providing a theory that can be applied to analyse and compare actual examples. Formulations have centered upon the expression of the photosynthetic rate (P)—light intensity (I) characteristic, with maximum rate (P_{max}), in relation to the varying surface intensity (I_o) and vertical light intensity gradient, the latter defined by some "average" or "effective" value of the vertical extinction coefficient (k_e). As a limiting case, a uniform population in the photosynthetic zone can be supposed, of density n. If the surface intensity I_o is sufficiently low so that light saturation of photosynthesis can be neglected, the formulation of the photosynthetic productivity per unit area (ΣP) is much simplified; examples are given by Riley (170), Sverdrup (215), and Vollenweider (244). Berge (3) has also used this simplification in computations for some sea areas at high latitudes. The more usual significance of light-saturation for ΣP has been calculated graphically by Ryther (175, 176) and in mathematical derivation by Talling (218). The central expression of Talling [(218) equation 3], using the present symbols, can be reduced to

$$\sum P = \frac{nP_{max}}{k_e}\left(\ln \frac{I_0}{0.5I_k}\right)$$

This expression was used to formulate other factors involved in the photosynthetic utilization of solar radiation by planktonic algae, and was applied to the characteristics of some specific populations in nature [Talling (218, 219)]. A parallel theory developed for the photosynthetic or growth kinetics of algal mass cultures has yielded expressions [Oorschot (155), equation 4; Tamiya *et al.* (223), equation 6] that can be re-interpreted in a similar form and applied over a wider range of the ratio I_o/I_k, namely

$$\sum P = \frac{nP_{max}}{k_e} \ln\left(\frac{I_0}{I_k} + 1\right)$$

These equations give a formal and simplified expression to several limitations of the integral photosynthesis that have often been discussed. The relation to the logarithm of the surface light intensity implies that the integral photosynthesis will be relatively insensitive to variations in I_o when this (and the I_o/I_k ratio) is high. Such insensitivity has been commented upon by various authors [e.g., Harvey *et al.* (75), Steemann Nielsen (201, 204), Talling (218)] who have considered the vertical displacement and enlargement of photosynthesis-depth profiles with increasing surface intensity.

Derivation of the photosynthetic productivity in nature from measurements on samples is clearly more difficult for a terrestrial plant cover. Some comparisons have been made between measurements of the photosynthetic behaviour of entire stands and their component leaves [e.g., Boysen-Jensen (20, 22), Heinicke & Childers (77)]; rate-intensity curves and diurnal changes of photosynthetic rate show less abrupt saturation at higher intensities in the dense stands. Transitional behaviour during stand growth is described

for rice plots (190, 216, 261). Similar consequences of self-shading effects in dense leaf systems have been discussed for various conifers [Bormann (14, 16), Kramer & Clark (108), Stålfelt (195)].

Several alternatives have been used in attempts to calculate indirectly the daily photosynthesis of a community in nature. As with phytoplankton [cf. Manning & Juday (123)], they have centered on the combination of the rate-intensity characteristic with the daily variation of incident illumination. In examples given by Boysen-Jensen (20, 21, 22), Larsen (113), and Polster (164) the characteristics from single leaves or twigs were used, and are not suitable for dense systems of overlapping leaves. Such a system, utilizing the matted growth of the moss *Homalothecium sericium*, was used directly by Romose (172) in his experiments. The results were combined with data on diurnal illumination at various seasons to estimate photosynthetic activity under natural conditions, using an average gradient for rate-intensity curves in relation to daily illumination-integrals (as lux-hours). A mean gradient was used in another way by de Wit (259, 260) to calculate theoretically the potential daily photosynthesis of dense plant cover from daily radiation-integrals; in the latter he distinguished two fractions, one being utilized in the calculation.

A more flexible theoretical treatment is described in some work of exceptional interest by Monsi and his associates (99, 139). The approach has many features in common with that outlined above for planktonic algae, and is based on the mathematical integration of photosynthetic rate with depth of plant cover. As in dense algal communities, the corresponding vertical light gradient can be related to the density of photosynthetic material. The stratification of the latter, and the associated light gradients, are illustrated for various natural communities, and discussed in relation to the transmission of individual leaves. The depth integration of rates yields an expression (99, equation 4) that can be transformed by appropriate substitution of symbols into a form very similar to that quoted for algal suspensions. The significance of the ratio P_{max} to k_e (the latter now related to leaf area index), and of the logarithmic function of the ratio I_o/I_k, can then be seen. The model is illustrated by data of Boysen-Jensen (20) on the photosynthesis of leaves and plant stands. Further and more direct comparisons with observed photosynthetic rates are needed to test the assumptions and simplifications involved.

Integrated yields of photosynthesis can be used for independent comparison with growth increments if both the carbon content of the latter and the various sources of loss (e.g., respiration) are known quantitatively. Few studies of photosynthesis under field conditions are sufficiently detailed to permit the comparison, but included among the few are those of Heinicke & Hoffman (78) and Heinicke & Childers (77) on *Pyrus malus*, Larsen (113, 114) on *Solanum* spp., Romose (172) on *Homalothecium sericium*, Thomas & Hill (227, 228) on several crop plants, and Tranquillini (233) on *Pinus cembra* [cf. also (55, 164)]. In these examples, except that of *Pinus cembra*, the agreement was good considering the problems involved, particularly the

one of obtaining estimates of respiration losses over long periods. Uncertainties concerning these and other losses have generally prevented the effective comparison of growth and photosynthetic rates for natural populations of planktonic or other algae [see, however, Sargent & Lantrip (183), Verduin (237)].

The calculation of growth increments per unit of leaf quantity (usually area) is the basis of a particularly successful form of growth analysis suitable for ecological application [see (7, 8, 34, 76, 247, 248), and particularly Watson (254, 255)]. The instantaneous rate of growth, defined as the "net assimilation rate" or "unit leaf rate" (E), can be related to the unit area productivity of the stand by the more variable factor of the leaf area index, and to the relative (specific) growth rate by the ratio of leaf area to total dry weight. Average values of E during a growing season often show a remarkably limited variation with species or latitude, although this is now known to be considerably broader than the "typical" range of 0.4 to 0.7 g./m.^{2} per week originally suggested by Heath & Gregory (76). Seasonal variation is described by Watson (254, 255); maxima of E may not coincide with the best development of leaf area index, thus limiting the unit area productivity. Another limitation of unit area yield, by an inverse relation between E and leaf area index, can also develop (255, 256); the optimum leaf area index for yield is therefore of much interest [Watson (254); cf. (99, 139)]. The average magnitude of E corresponds roughly [Thomas (226)] with that expected from the usual order of the net photosynthetic capacity of leaves at light saturation, about 0.7 to 1.7 g. carbohydrate/m.^{2} per hr. [cf. the data of Larsen (113, 114) for *Solanum* spp.]. Although the net assimilation rate reflects factors other than the net photosynthetic rates of leaves (e.g., respiration of non-photosynthetic organs, ash content), the relation of these quantities is sufficiently close to make their combined study in field conditions [see Iwaki (91)] most desirable. There is, for example, a suggestive parallel of a modified logarithmic relationship established between net assimilation rate and the daylight factor [Blackman & Black (6), Blackman & Wilson (8)], and a similar relationship is indicated by theory [cf. Talling (218) Fig. 6] between photosynthetic productivity per unit area and light intensity in a dense homogeneous cover.

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PHOTOSYNTHESIS[1,2,3]

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Reviews like this one on photosynthesis have been rather abundant in recent years (1 to 5). An encyclopedic coverage of photosynthesis and related areas of plant physiology comprising a number of authoritative articles was recently published (6). Gaffron (7) has written an excellent introduction to the field and the proceedings of three meetings dealing with photosynthesis have become available (8, 9, 10). With this variety of readily available material, the authors felt no compulsion to make this review all-inclusive. Therefore, much interesting literature has doubtless been ignored or treated fleetingly.

Chloroplast Structure

The fine structure of the photosynthetic apparatus of plants, including algae and photosynthetic bacteria, has been reviewed recently by Wolken (2), Mercer (11), Spikes & Mayne (5), and Thomas (12).

Additional evidence on the association of pigments with the lamellar system has been provided subsequently by several authors. Shatkin (13), with the aid of the electron microscope, recognized the presence of lamellae in the fraction of *Anabena variabilis* extracts that contained all the chlorophyll and carotenoids. The phycocyanin, however, remained in the soluble phase. Brody & Vatter (14) correlated evidence obtained by electron and light absorption microscopy on fixed whole cells and concluded that the phycobilins, as well as the chlorophyll, seem to be associated with the lamellae of the plastid in *Porphyridium cruentum*.

Further evidence on the association of chlorophyll, proteins, and lipids with lamellar structures in chloroplasts was reported by Greenblatt *et al.* (15) who studied living *Euglena gracilis* cells and chloroplasts extruded from them with monochromatic (435 mμ) light absorption microscopy. Exposure of the chloroplasts to lipase and lecithinase appeared to increase the number of pigmented lamellae, indicating a lipid interface binds adjacent lamellae. The proteolytic enzymes trypsin and papain disrupted the pigmented lamellae.

[1] The survey of literature for this review was concluded in November 1960.

[2] The following abbreviations are used: CMU [3-(4-chlorophenyl)-1,1-dimethylurea]; DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea]; DCPIP (dichlorophenolindophenol); DNP (2-4,dinitrophenol); FAD (flavine adenine dinucleotide); FMN (flavin mononucleotide); *g* (in practice, a constant times the ratio of microwave frequency over the magnetic field strength at which electron spin resonance signals are found); PCMB (para-chloromercuribenzoate); PMS (phenazine methosulfate); P700 (pigment absorbing at 700 mμ).

[3] Preparation of this paper was supported by the National Institute of Health (RG 6692) and the Air Force Office of Scientific Research [AF 49(638)-947].

Conclusive evidence on the localization of photosynthetic pigments within the lamellae is still lacking. The large differences in absorption spectra between the intact cell and extracted chlorophylls leaves little room to doubt that chlorophyll molecules in the plastid are in some organized state. Trurnit & Colmano (16) have simulated the absorption spectrum of chlorophylls *in vivo* by monolayers of chlorophyll at water/air and water/oil interfaces. The authors regarded these data as supporting the hypothesis that chlorophyll is present in a film state in the lamellae.

In a study of the chloroplast of the green alga *Chlamydomonas reinhardi*, Sager & Palade (17) recognized that the basic unit of the lamellar system, i.e. a single lamella, is a flattened vesicle which they designated as a disc. Furthermore, they observed the occurrence of stacks of discs that resembled the organization found in grana of higher plant chloroplasts. They postulated that "the disc may represent a general and pervasive feature of chloroplast organization."

More recently, descriptions of single lamellae as discs or flattened sacs and recognition of the latter as structural units of the lamellar system have appeared in the works of Lefort (18) on the blue-green algae *Oscillatoria* and *Phormidium*, by Shatkin (13) on the blue-green alga *Anabena*, and Brody & Vatter (14) with the red alga *Porphyridium;* however, none of these authors mentions the occurrence of stacks of discs. Some observations on the fine structure of the lamellar systems of higher plant chloroplasts have recently been reported by Menke (19), Lefort (20), Lance (21), and Schidlovsky (22). These authors agree also that the morphology of a single lamella of the chloroplast of a higher plant is best described by analogy to a flattened sac or disc. By following the process of greening in dark-grown bean plants, the last named author concluded that grana arise by a process of fusion or close juxtaposition of flattened sacs. Other observations of Schidlovsky suggest that the lamellar systems of organisms lacking "grana" (such as *Euglena*, *Scenedesmus*, and *Ochromonas*) exhibit a comparable juxtaposition of sacs. However, in this case it extends over the full length of the lamellae.

Quantum Yield

In a recent review by one of us (23) the conclusion was reached that in photosynthesis, regardless of the type of organism and the nature of the oxidant or the reductant, two photons seem to be required per hydrogen transfer. More recently, Warburg, Krippahl & Gattung (24) again reported single vessel manometric measurements of O_2 exchange. A large side arm contained a carbonate buffer mixture to maintain a constant CO_2 pressure and carbonic anhydrase to speed up equilibration (25). For the observation of computed quantum numbers as low as 3 $h\nu/O_2$ the use of a blue-green compensating light and a high partial pressure of CO_2 were prerequisite. This number was only found with very weak light ($I \leq 2$ $\mu lh\nu$/min.) and doubled in the increment between 2 and 5 $\mu lh\nu$/min. These results therefore appear entirely comparable to those of earlier published experiments of

Warburg *et al.* under high pCO_2 (26) and hence are open to a scrutinization similar to that given earlier (23, page 609).

Duysens (27, 28) argued that the efficiency of photosynthesis cannot exceed 70 per cent. To the authors, his argument, which is based on the second law of thermodynamics, is open to question.

"R Factor"

In a series of papers, Miyachi and co-workers (29 to 39) investigated the post-illumination CO_2 fixation occurring in green algae. They divided the fixation into two types. The first was a normal dark fixation, having no relation to the photosynthetic fixation of CO_2 and therefore only indirectly affected by pre-illumination. The system responsible for this fixation was designated *r* and the capacity of the system (*r* level) was assayed by the amount of CO_2 fixed in a 20-min. period subsequent to illumination. The second type of fixation was thought to represent a post-illumination survival of the photosynthetic reductant and therefore entirely comparable to CO_2 fixation occurring in the light. The concentration of this reductant, termed *R*, was measured by the amount of CO_2 fixed in 30 sec. after illumination. In this time period *R* had completely decayed. Under appropriate conditions *R* was very much higher than *r*. It was found that the *r* level was generally 2 to 10 times larger when the cells were in oxygen as compared to nitrogen. Upon changing from nitrogen to oxygen the *r* level shows a sharp rise with a slow decay to its steady-state level in oxygen (32). Upon illumination of anaerobic cells the *r* level showed a slight depression, then climbed to a steady-state level. The steady-state *r* level was reached more quickly than the steady-state *R* level. At the cessation of illumination, *r* quickly rose and then declined to a lower steady-state level. When the cells were allowed to fix $C^{14}O_2$ anaerobically in the dark and then given a short flash of light (less than 10 sec.) a $C^{14}O_2$ burst was observed (32).

The time-course of formation of *R* showed two inductions (32), and 30 to 40 min. were required to attain a maximal *R*. The effect of various compounds on the formation and breakdown of *R* was studied. Oxidizing agents such as oxygen, hydrogen peroxide, quinone, and DCPIP lowered the steady-state *R* level but had no effect on the formation of *R*. Cyanide did not retard formation of *R* but lowered the steady-state *R* level and inhibited the reaction of *R* with CO_2. Azide and hydroxylamine inhibited the rate of formation of *R*. At low temperatures (5–7°C.) these inhibitors accelerated decay of *R* but at 25°C. they either had no effect or, in the case of hydroxylamine, retarded the decay. Azide and hydroxylamine inhibited the reaction of *R* with CO_2 but iodoacetate and *p*-chloromercuribenzoate had very little effect. The decay of *R* in the dark was accelerated by *p*-chloromercuribenzoate and by arsenite. Iodoacetic acid did not affect the decay of *R* but inhibited its rate of formation.

Since pyridine nucleotides are generally considered to be the CO_2 reductant, Miyachi *et al.* (38) and Oh-hama & Miyachi (39) attempted to cor-

relate the concentrations of *R* and reduced pyridine nucleotides. No parallelism was found; for example, illumination of dark-incubated cells produced an increase of *R* equivalent to 25×10^{-7} moles CO_2 fixed per gram dry cells while the increment of reduced pyridine nucleotides (DPNH+TPNH) was 0.5 to 2.0×10^{-8} moles per gram. After illumination the *R* level dropped abruptly, whereas TPNH and DPNH concentrations declined slowly.

The authors postulated that *R* is a reducing agent capable of reducing CO_2 to the carbohydrate level and that the bicarbonate fixed by *R* is reduced. However, no evidence for the reduction of CO_2 by *R* is presented. The earlier papers by Benson & Calvin (40) and Benson *et al.* (41) indicated that a very small amount of the CO_2 fixed subsequent to illumination is actually reduced. Of the CO_2 fixed in 5 min. after illumination (corresponding more to *r* than *R*) only about 3 per cent is reduced. Thus, the premise that *R* is the photosynthetic reductant seems premature.

Effect of light on pyridine nucleotide concentrations.—Oh-Hama & Miyachi (35, 39) utilized enzymic assays to measure the effect of illumination on the concentration of oxidized and reduced pyridine nucleotides in *Chlorella*. Illumination in the absence of CO_2 following a period of dark anaerobiosis resulted in an increase in TPN and TPNH and a decrease in DPN and DPNH. The authors suggest that a photochemical conversion of DPN to TPN occurred under these conditions. When the experiment was performed aerobically, DPN declined in the light, DPNH did not change, and TPN and TPNH increased. In the dark, the presence of oxygen caused a rapid loss of DPNH but had little influence on the levels of DPN, TPN, or TPNH. Experiments were also carried out in the presence of CO_2. From the changes observed the authors conclude that TPNH, but not DPNH, is participating in some way in the mechanism of photosynthesis.

Electron Spin Resonance Studies

Since it is likely that electrons are moved one at a time in photosynthesis, intermediates such as triplet states, free radicals or conduction band electrons could be expected to be involved. An increasing amount of work has been devoted to the detection of these unpaired electrons with the electron spin resonance (ESR) method. Presently the interpretation of signals is still ambiguous. Triplet state electrons in a single molecule appear to be undetectable (42, 43), and Commoner (43) has cautioned against interpreting signals arising at liquid nitrogen temperature as unique for semi-conductor mechanisms.

Treharne *et al.* (44) have reported spin resonance signals in *Chlorella* that are related to manganese concentration.

Using an electron paramagnetic (spin) resonance spectrometer of improved design, Commoner (43) has observed two light-responsive signals in chloroplasts and in *Chlorella*. One signal was present in the dark and increased in intensity during illumination. This signal was centered at $g = 2.005$, had a half-width of about 19 gauss, and showed distinctive hyper-

fine splitting. Commoner suggests that this signal is due to an organic free radical, the locale of the unpaired electron being a resonance system of four equivalent carbons, each of which possesses a proton. The magnitude of this signal was decreased by 5 per cent carbon dioxide in air, either in dark or light. The onset and decay of the response to illumination was rather slow ($t\ \frac{1}{2} \simeq 10$ sec.). The signal responded to light intensity in a fashion similar to photosynthesis, saturating at 20,000 lux when the temperature was maintained at 29.5°C. but reduced to 10,000 lux at 12.8°C.

Upon illumination of *Chlorella* at lower temperatures, or of well washed or dialyzed chloroplasts, the absorption on the low-g side of the 2.005 peak increased. Subtraction of the dark absorption from the light absorption resulted in another peak at $g = 2.002$ to 2.003 without hyperfine structure. The signal is enhanced by CO_2 and exhibits a rather peculiar response to light intensity. At 12.8°C. (it is not detected at 30°C.) saturation was not achieved until 60,000 lux. Commoner suggests the 2.002 signal is the precursor of the 2.005. He reasons that in fresh chloroplasts or in algae at normal temperatures 2.002 does not reach a large steady state concentration because of fast transfer to 2.005. Since the rate of decay of the product yielding the 2.005 is quite slow, it will accumulate. Only when transfer from 2.002 to 2.005 is slowed down (by low temperature, washed chloroplasts, or by saturating 2.005) will the 2.002 signal be measurable.

The signals obtained from a normal green strain of *Euglena* were essentially identical to those of *Chlorella* and chloroplasts. In contrast, a colorless *Euglena* mutant exhibited only a peak at $g = 2.006$ without hyperfine structure and not responding to illumination. *Rhodospirillum rubrum* gave a single signal at $g = 2.003$ which responded to light.

Calvin and co-workers (42) have observed ESR signals in photosynthetic organisms. The light-induced chloroplast signal was not resolved into the two components noted by Commoner, but the decay kinetics indicated at least two different kinds of unpaired electrons. The rate of formation of the signal appeared to be very rapid both at 25°C. and at −150°C. At the lower temperature the signal did not fall when illumination ceased, indicating that thermal reactions mediated the decay. The very rapid rise of the signal, even at −150°C., suggests that the free radical is formed photochemically. The data of Commoner and Calvin do not appear to be in exact agreement on this point.

Calvin and co-workers measured the action spectrum for electron spin resonance signal production in chloroplasts and *Rhodospirillum rubrum*. Both spectra showed a maximum located on the long wavelength side of the red or infrared absorption band and a minimum almost coinciding with the absorption peak. It appears that the term action spectrum is incorrectly applied, since Calvin states the samples were totally absorbing. Hence this spectrum is one of relative efficiency versus wavelength. Calvin has discussed the possible artifacts arising from this sort of measurement. The reviewers feel that a two-quanta mechanism similar to that discussed later in this

review could explain these data. In this hypothesis one pigment, on absorbing light, would produce these spins but another (different) pigment would have to be excited to further process the spins (and thus hasten their decay). A greater steady-state concentration of unpaired spins would result from exciting the former pigment without exciting the latter.

Light Minus Dark Spectra

Illumination can provoke quantitative conversions of chlorophyll molecules in solution (45). Only minute changes have been detected in the plant, the greatest occurring in purple bacteria (46). The detection of the small, light-induced absorption changes of carotenoids, chlorophylls, and cytochromes requires rather sensitive techniques. Besides sensitivity, the technique of measurement requires avoidance of interference between the detecting illumination—used to monitor the absorption—and the activating light, or the fluorescence and chemiluminescence it induces. This interference can be avoided by using complementary filters in the two beams (color separation) which tends to restrict the wavelength region surveyed. No such restriction is met if the two beams are alternated (time separation), but this necessarily introduces a delay between induction and observation of the effects. In the following we will discuss the results of several studies in this field.

Carotenoids.—Duysens (46), using a split beam difference apparatus and color separation of actinic and detecting lights, observed, upon illumination of a *Chlorella* suspension, bands of decreased absorption around 420 and 480 mμ accompanied by an increase at 515 mμ (47). The 478/515 shift was attributed to a band shift of an unknown pigment. It was not found in the red alga *Porphyridium* and Duysens was also unable to observe a concomitant change in the red part of the spectrum. On the basis of the influence of various poisons and the enhancing effect of anaerobiosis (48), he concluded that chlorophyll was not involved in this change, but rather some intermediate in the process of CO_2 reduction. Spruit (49) used a method very similar to that of Duysens but observed a stimulation by oxygen rather than a decrease of the light-induced change at 515 mμ. In the dark, oxygen added to an anaerobic suspension of *Chlorella* caused a decrease of optical density at this wavelength. Bell (50) concluded from experiments carried out in a similar fashion that the 515 mμ shift was due to a side reaction in photosynthesis; he observed no influence of urethane or of the absence of CO_2, a decrease of absorbence under anaerobiosis or prolonged (5 hr.) illumination, and saturation in relatively weak light.

Strehler & Lynch (51, 52, 53) studied the increase of absorption at 515 mμ with a split beam apparatus. There was a transitory overshoot before the absorption reached a steady-state value (after about a minute). On darkening there was a transitory overshoot (below the steady-state dark level) before the absorption returned to the original level. The induction peak at the start of illumination required far less (1/10) light to develop than did the

steady-state shift. With a flow system, characterized by a long delay ($\simeq$ 1 sec.) between illumination and observation, the 475/515 system showed the usual band shape but appeared inverted: in the light, absorption was decreased at 515 mμ and increased at 475 mμ. The same inversion was found for the 650/660 mμ shift observed by these authors (cf. below). With a modified phosphoroscope arrangement in which the delay between illumination and measurement was only 0.1 sec., no such inversion of the effects was observed. Measurements with the flow system showed that the sign of the absorption shift was inverted only at higher temperatures and not at lower ones (4°C.). These observations indicate the existence of dark reaction mechanisms capable of performing changes of the 475/515 mμ pigment similar, as well as opposite to, the ones induced by light. Chance and co-workers used, in addition to a regular split beam setup, an apparatus in which two beams of different wavelength alternately (60 c.p.s.) passed through the sample. One of these is selected in an inert part of the spectrum and serves as a reference. Separation of actinic and detecting lights was accomplished with complementary filters. Chance & Strehler (54) noticed that oxygenation of anaerobic *Chlorella* cells yielded absorption changes very similar to the ones induced by light. In such anaerobic algae extremely weak red light (0.3 ft. c.) slowly induced a sizeable fraction of the maximum possible (steady-state) 475/515 shift. This shift was interpreted as a result of the algae becoming more oxidized, possibly as an effect of the photosynthetic oxidant. If, after establishment of the steady-state shift, the intensity was increased, a further absorption change took place initially, followed by a partial return to the level in weak light. Subsequent darkening yielded a sudden partial drop of the 515 mμ absorption followed by a slow return to the initial anaerobic level along with the depletion of oxygen by respiratory uptake. Aerobic cells, presumably because most of the 475/515 system is already in the oxidized state, showed a much smaller effect of light which moreover required a hundredfold higher intensity for half saturation. The authors concluded that two effects with very similar spectral manifestations are involved: a low intensity effect identical with oxygenation and an effect of high intensity that cannot be provoked by oxygen alone.

Chance & Sager (55) studied oxidized minus reduced, as well as light minus dark, spectra with a pale green mutant of *Chlamydomonas* with a carotenoid content of only 0.002, and a chlorophyll content about 0.1 of that of the parent strain. This mutant, which was photosynthetically active although sensitive to prolonged illumination (56), did not show any 475/515 mμ absorption change as did normal green cells, including the parent strain. This was considered to support the assumption that the 475/515 shift involves conversion of a carotenoid pigment and that carotenoids play only an indirect role in photosynthesis such as protection against photooxidation (57). Kok (58, 59) studied the absorption changes in this part of the spectrum with his technique as described below. In green cells the positive change had a complex bandshape—showing up to three maxima. In diatoms the effects

were even more complex and showed a main positive peak at 560 mμ at the long wavelength side of the predominant carotenoid fucoxanthol. These data were thought to indicate that changes of carotenoid pigments underlie the absorption shifts. Actinic flashes (given with a rate of 10 to 20 per second) invariably induced a short-lived increase of absorption at 515 mμ, but evidently due to a back reaction occurring during the relatively long interposed dark periods, the background absorption level at 515 mμ decreased during illumination (after an initial positive transition during some 10 sec.). Removal of the actinic illumination temporarily produced a further absorption decrease (which revealed the accelerated dark back reaction) after which the zero level was again attained in the course of a few minutes. Differences in time course and response to illumination led to the conclusion that there was no direct relation between the 515 change and the shifts observed in the red part of the spectrum. Also, Coleman & Rabinowitch (60), using continuous actinic illumination, observed a difference of intensity response between "carotenoid" and "chlorophyll" effects, in fact a difference of intensity response between the 480 and 520 band.

An extensive series of notes and papers by Witt and co-workers which were recently surveyed, but not in English (refer to 61, 62 for original literature), deals with rather unique observations of the absorption changes between 400 and 570 mμ. A thin layer of sample material was located in front of a photomultiplier cell and illuminated with monochromatic detecting light. Flashing actinic illumination ($\lambda > 600$ mμ) was generated with flash tubes or, for longer flash-times with rotating discs or shutters. Direct oscillographic observation allowed a study of the time course of absorption during and after illumination even with a single brief flash (instrumental resolution: $\Delta I/I$: $2 \cdot 10^{-4}$, band width: 0 to 10 kc.). Witt and co-workers observed four different types of light-induced absorption changes, all of the order $\Delta I/I \simeq 10^{-3}$, which we will briefly recapitulate. Type "zero" consisted of an absorption decrease between 400 and 450 mμ with an unsharp maximum around 430 mμ and a simultaneous increase of absorption at all wavelengths beyond 450 mμ. This change was observed with solutions of chlorophylls or pheophytins in pyridine, with chlorophyll precipitated on protein, all types of plant material extracted with acetone, treated with digitonin, or heated above 65°C. The lifetime of this change was extremely short (half-time $< 10^{-5}$ sec.) and unaffected by temperature down to −160°C. The effect was ascribed to a small percentage of the chlorophyll molecules brought into the triplet excitation state (63, 64, 65).

The change denoted as "Type 1" appeared as a broad area of absorption decrease (400 to 475 mμ) and a rather wide band of increased absorption centered around 520 mμ. The decay of the absorption change after the flash was some tenfold slower ($t_{\frac{1}{2}} \sim 10^{-4}$ sec.) than that of the Type O change. Temperature (−200 to +60°C.) did not affect this lifetime. Changes of Type 1 were observed with all types of aerobic photosynthetic organisms. It disappeared after disrupting the structure with detergents but remained in sam-

ples that were briefly heated to 60°C. or extracted with hexane (to remove carotenoids). Addition of the paramagnetic gases O_2 and NO at a pressure of one atmosphere decreased the magnitude of the effect to the vanishing point. Witt interpreted this Type 1 effect as the conversion of the triplet state of chlorophyll-*a* (formed in change Type O which presumably precedes Type 1) into a more stable derivative. He assumed a reaction with a partner molecule present in close proximity on the structure, so that no temperature effects occur. The effect of paramagnetic gases is easily explained on the basis of their well known property to quench the triplet excited state. The argument for assuming a transformation of a small fraction of the chlorophyll-*a* was based on the universal occurrence of the Type 1 change (i.e., also in plant material which does not contain chlorophyll-*b* or from which carotenoid is extracted).

The changes denoted as "Type 2" consist of two bands of absorption decrease at 420 and 475 mμ respectively, along with an increase at 515 mμ. These appear to correspond to the ones observed by the various other workers (see above). They are only observed in green algae and leaves (or chloroplasts) of higher plants and are not induced by far-red light ($\lambda > 700$ mμ). The changes occur immediately ($\leq 10^{-4}$ sec.) upon illumination and decay in subsequent darkness with a rate dependent upon temperature (in whole cells $t_{\frac{1}{2}}$ at 20°C.$\simeq$0.01 sec., $t_{\frac{1}{2}}$ at 0°C.$\simeq$0.06 sec.). This decay was slowed down considerably if the flash was given after a long dark period. The magnitude of the effect induced by brief flashes is independent of temperature between 0 and 45°C. (beyond the latter limit the system becomes inactive). It is unaffected by the presence of KCN or quinone (reagents which inhibit CO_2 assimilation) or the absence of CO_2, O_2, or Mn, but is decreased by urethane. The effect increases with light intensity until it begins to saturate at a flash (2×10^{-4}sec.) intensity of $\sim 5 \times 10^4$ erg cm.$^{-2}$ sec.$^{-1}$. This intensity is an order of magnitude lower than required to saturate the changes of Type 1 (and also, as far as the present reviewers can estimate, to approach flash saturation in photosynthetic oxygen evolution).

A study of the effect of flash time (over a range between 10^{-5} sec. to 10 sec.), revealed a complex time course of the change. An initial spike was followed by a slow, temperature-dependent build-up. After a few tenths of a second (20°C.) a final level was attained that was some twofold higher than the initial spike. The second "slow" phase is absent in manganese-deficient cells and at low temperature. It also fails to occur after a prolonged dark-time, in which case the initial fast rise of absorption at 515 mμ decays—even in the light. But after a preillumination period of sufficient length, the second phase occurs immediately, rising so rapidly that it tends to mask the fast initial spike of the first phase. This pattern of behaviour resembles the aerobic induction in photosynthesis (66, 67) and the oxygen burst at the onset of illumination (68, 69). Together with the temperature-independent "maximum change per brief flash," the dark decay times, and the two-phase build-up in the light, these data indicate a close correlation between the

kinetics of the absorption changes and that of photosynthetic oxygen evolution. Like other workers, however, Witt & Moraw (70) concluded that at least one chemical conversion separated the 515 mμ effects from the primary photochemical events. Evidence for this was, for instance, their observation under appropriate conditions of a continuation of the light-induced change after cessation of illumination. Occasionally it was also found that, after illumination, the 515 mμ absorption decayed below the original dark level; but the effects of oxygen reported by other workers could not be observed. One can probably ascribe this last point of discrepancy to the fact that the demanding technique of Witt *et al.* required a relatively strong detecting beam. This beam by itself may have provoked the oxygen and low light effects (observed by others) so that the influence of external O_2 escaped measurement.

Witt *et al.* also studied the absorption changes of Type 2 with isolated chloroplasts. Activity was lost after hexane extraction of lyophilised chloroplasts and restored by readdition of the extracted material. The optimum pH was between 6 and 7. Unlike whole cells, chloroplasts show only the first (fast) phase of absorption change. At the same time no more effects of preillumination are observed. The decay of the change induced by a brief flash is very slow in washed chloroplasts ($t\ \frac{1}{2} \simeq 0.1$ sec. compared to $\simeq 0.01$ sec. in whole cells). Dyes characterized by oxido-reduction potentials ranging between $E_o' = 0.0$ volt to $+0.26$ volt accelerated this decay, in a concentration-dependent fashion. For instance, with $3 \times 10^{-5} M$ DCPIP (the most effective compound) $t\ \frac{1}{2}$ was 0.01 sec. and with $5 \times 10^{-3} M$ the half life became as small as 0.4 msec. Reduced dyes did not influence the decay. On the basis of these data Witt & Müller (71) assumed that reaction:

$$X_{red}(515) + S_{ox} \xrightarrow{k} X_{ox}(420, 475) + S_{red} \cdots \qquad 1.$$

described the reconversion in the dark of X_{red} (515) formed during the light flash. Measurements of the decay time as a function of dye concentration and temperature yielded, for the case of DCPIP, a value for $k \simeq 2.7 \times 10^6$ l./mole-sec. and an activation energy of 7.4 kcal./mole.

The inability of dyes with an $E_o' < 0.0$ v. to yield acceleration of the decay was thought to correlate with Wessels' (72) observation that such dyes were not reduced in the Hill reaction. Conversely it was concluded that the redox system X_{red}/X_{ox} had an E_o' value of about 0.0 v. However, it appeared that redox potential is not the sole important characteristic since activity of the various dyes, even if added in the same concentration, varied greatly. *M*-cresol indophenol, having about the same potential as DCPIP was fully inactive—as were also both the Hill reagents, *p*-benzoquinone and ferricyanide. Also, other evidence discussed in this article (e.g., the likelihood of oxidation rather than reduction of the 475 pigment) in the reviewers' opinion yields a tentative character to the proposed reaction mechanism 1. Witt *et al.* were hesitant to ascribe their "Type 2" effects to changes of carotenoid. The

main argument for this was the closely parallel behavior of the 420 mμ shift. Other workers did not report such strict parallelism (58, 60).

Light-induced changes of carotenoid pigments are not restricted to aerobic photosynthesis. Smith & Ramirez (73) and Chance (74) analyzed the complex light minus dark and oxidized minus reduced spectra found with the photosynthetic bacteria *Rhodospirillum rubrum*, *Rhodopseudomonas spheroides* and carotenoid-deficient mutants of the latter. In carotenoid-containing cells, oxygenation as well as illumination yielded a rapid (5 to 10 per cent) decrease of the absorption bands characteristic for these pigments and this was accompanied by an increase of absorption at longer wavelength. Similar observations made by Arnold & Clayton (75) will be discussed later.

Cytochromes.—The literature pertaining to the role of cytochromes in aerobic and in bacterial photosynthesis has been reviewed by Smith & Chance (79), Frenkel (80), and Gest & Kamen (81).

Recently Katoh (82, 83, 84) isolated and crystallized a *c* type cytochrome typical for the plastids of the red alga *Porphyra tenera*. The reduced form had absorption bands at 417, 521, and 553 mμ. Its E_o' value at pH 7 was 0.335 v. and changed in a linear fashion with pH, an uncommon feature among this type of compounds. A rapid, cyanide-insensitive oxidation of the pigment occurred in illuminated preparations, whereas in the dark a low and cyanide-sensitive rate of cytochrome oxidase activity was found. The cytochrome could be reduced faster by the extracts with TPNH than with DPNH and not at all by succinate (which the intact cells rapidly oxidize). The author suggests that the possible function of this cytochrome is in photosynthesis. Nishimura (85) isolated from green (but not from heterotrophically grown and colorless) *Euglena gracilis* a *c* type cytochrome with absorption bands in the reduced form at 417, 523, and 552.5 mμ. Its E_o' at pH 7 was estimated to be 0.36 v. and showed a similar pH dependence as cytochrome-*f* [pK 8.3, cf. Davenport & Hill, (86)]. Nishimura used 0.409 v. for the ferro-ferri cyanide couple, whereas Davenport & Hill (86) used 0.43 v. in their titration of cytochrome-*f*. Based on the latter value, one arrives at $E_o' = 0.381$ v. for this *Euglena* pigment, the highest potential so far reported for a cytochrome.

There is an increasing amount of data that all photosynthetic machinery comprises at least one or two specific cytochromes. However, the difference spectroscopic evidence collected with green algae and leaf material so far has lent little or no support to a direct role of these catalysts in the primary events of photosynthesis. The light-induced change of cytochromes-*f* and -b_6 observed in green cells by James & Leech (87) [like the earlier observations of Lundgårdh (88)] appear more as secondary effects from a kinetic viewpoint.

The fast, light-induced, negative shift at 420 mμ found in green cells by all workers is not accompanied by a simultaneous increase at shorter wavelengths, as would be expected if the Soret band of cytochrome-*f* shifted upon oxidation (cf. Fig. 1); neither was the band at 553 mμ consistently observed. A clarification of the role of cytochromes in green plants seems definitely

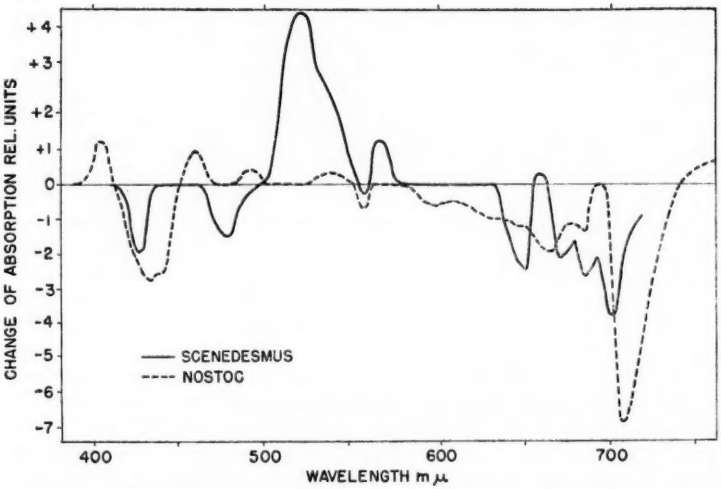

FIG. 1. Two examples of photosynthetic difference spectra obtained, with a green (solid line) and a blue-green alga (broken line). These particular spectra illustrate most of the bands observed, but dependent upon material and conditions their relative magnitude can greatly vary.

needed. However, as also shown in Figure 1, in blue-green and red algae a distinct bleaching occurs around 553 and 420 mμ together with an absorption increase below 410 mμ. On the long wavelength side of the Soret band (approx. 430 mμ), a second absorption shift occurred which Kok correlated with a bleaching of the blue band of P700 (pigment absorbing at 700 mμ). Duysens (89) ascribed these changes to oxidation of cytochrome-*f* by light—possibly the cytochrome described by Katoh is the one involved.

That at least one cytochrome plays a specific role in photosynthetic electron transport in bacterial photosynthesis seems well established (27, 73, 80, 81, 90).

Chlorophylls.—Strehler & Lynch (51, 52, 53), using a time separation method (flow system) with spinach chloroplasts, observed upon illumination a decrease of absorbence at 650 mμ accompanied with a slight increase at 660 mμ as the major effect in the red. The most obvious interpretation of this shift was that a small fraction of the chlorophyll-*b* underwent a temporary bleaching and a red shift of its absorption band. From differences in saturation intensity and time course, the authors concluded no direct correlation between the 650 and the 520 absorption shifts could be made. Coleman, Holt & Rabinowitch (98), using an apparatus similar to that used by Duysens, observed a band of decreased absorption at 680 mμ with *Chlorella*. Coleman & Rabinowitch (60) measured complete difference spectra (380 to

730 mμ) with *Chlorella* and various intensities of actinic light. They found that the effects in the red appeared only at intensities higher than those required to provoke (or even saturate some) shifts in the short wavelength regions. Flanked by smaller bands at 650 and 700 mμ, the 680 band was the most pronounced one, the maximum effect corresponded to the bleaching of about one chlorophyll molecule per 400 present. It was tentatively ascribed to a reductive bleaching of chlorophyll [Krasnovsky's "eosinophyll" (4)].

Kok (58, 59, 99, 100) applied a fast technique of time separation in which the actinic light was given as brief flashes. In one arrangement the flashes were given at about 0.1 sec. intervals, presumably long enough to allow the dark reaction(s) to reconvert a substantial part of the photoproducts. By appropriate gating of the photosignal, the absorption level during any two points of the dark-time could be compared, in order to detect intermediates that undergo cyclic color changes. A reference beam, which memorized the absorption level without actinic light was used to follow the background absorption level upon which these short-lived changes were superimposed. In green plants a number of absorption shifts were observed in the red which comprised bands at 650 (−), 660 (+), 680 (−), and 700 to 705 (−) mμ. Under the particular conditions of the experiments, the half-life time of all these absorption changes was on the order of 5 to 7 msec. The shift slightly beyond 700 mμ occurred in all photosynthetic organisms investigated (101) and was especially pronounced in blue-green and red algae. An absorption band slightly beyond the red band of chlorophyll-*a* might be the endpoint of energy transfer in photosynthesis and further work was therefore largely concentrated on this shift. The flash induced, dark-reversible bleaching effect appeared to be sensitized mainly by chlorophyll-*a* and possibly carotenoids. The maximum conversion—if interpreted as the bleaching of a chlorophyll—corresponded to about 1/400 of the total chlorophyll. The quantum yield of pigment turnover in *Anacystis* was estimated to be of the order of unity, indicating that a major fraction of light absorbed by chlorophyll-*a* could pass through transformation of P700 (102) and that a primary photochemical event was involved. The effect proved rather insensitive to most inhibitors of photosynthesis, survived lyophylisation and rehydration, extraction with water (i.e., removal of phycocyanin in *Anacystis*) or hexane (removal of carotenoid, coenzyme Q, etc.), sonic oscillation or treatment with detergents, and removal of 85 per cent of the chlorophyll (103).

The photochemical bleaching, however, proved not the only conversion of P700 driven by light. Kok & Gott (102) observed that the reverse reaction—regeneration of the pigment—although also occurring in the dark, was stimulated by illumination. This restoration did not become apparent immediately after the flash and thus seemed to require the mediation of a dark step. Activation of this "positive effect" was mainly via an accessory pigment (e.g., phycocyanin) including a small fraction of the chlorophyll-*a*. Two antagonistic reactions, both driven by light, in addition to dark steps, therefore appeared to determine the momentary concentration of P700. In con-

trast to the stability of the negative (chlorophyll-*a* sensitized) effect, the "positive" (accessory pigment) effect proved extremely sensitive to aging, heating, extraction, sonication, etc. It was reduced to half in $10^{-7}M$ DCMU, $10^{-4}M$ hydroxylamine, or $10^{-4}M$ dinitrophenol, but was insensitive to azide and KCN [Kok & Hoch (104)]. The photosynthetic oxygen evolution step responds in a very similar fashion to these poisons and on first sight the two appear correlated. Although no way was available to estimate the rate of pigment turnover in the positive effect, Kok & Hoch assumed it to be of the same order as that of the negative effect.

Bacterial chlorophylls.—Goedheer *et al.* (91) assumed that the reversible bleaching of chlorophyll in methanolic solution by ferric chloride, discovered by Rabinowitch & Weiss (92), was a true reversible oxidation and measured the redox potentials characterizing 50 per cent bleaching of the pigments. For chlorophyll-*a* and -*b*, values of +0.645 v. and +0.680 v. were found whereas bacteriochlorophyll and bacterioviridin showed a lower value: +0.55 v. Probably more directly correlated with *in vivo* processes are the changes of absorption with redox potential that occurred in the chromatophores of purple bacteria. (Only irreversible oxidative bleaching was observed with extracts of green bacteria or chloroplast suspensions.) Besides a small bandshift at 805 mμ, Goedheer (93) observed a bleaching of the longest wavelength absorption band of the various bacteriochlorophyll protein complexes as the main effect of oxidation. Depending upon the type of bacteria this band is located between 870 and 890 mμ and was denoted as "B890" by Duysens (46). In extracts from *Rhodospirillum rubrum*, the band was halfway bleached at a potential of +0.65 v. Certain difficulties in these measurements are evident because of the slow response and only partial reversibility. B890, shown to be the only one to fluoresce regardless of which band absorbed exciting light (46), possibly plays a role similar to the one of chlorophyll-*a* in aerobic photosynthesis. The relative fraction of bacteriochlorophyll present in the "890" form varies widely between species and also depends upon culturing conditions. In *R. rubrum* practically all pigment is in the "890" form, whereas in *Chromatium* this may account for less than 10 per cent. In either case, Duysens (46) observed (in addition to other effects) a light-induced (steady-state) decrease of this band amounting to about 4 per cent of the total absorbency. The change was larger and required a much lower intensity if suspensions of *R. rubrum* were not quite anaerobic and occurred presumably only after a cytochrome partner in the system was fully oxidized (94). This, and the resemblance between the light-induced and oxygen-induced absorption changes, indicated that during illumination part of pigment B890 becomes oxidized [a fraction corresponding to that oxidized, by poising the system at a redox potential of +0.515 v. according to Goedheer (93) or +0.44 v. according to Duysens (94)]. Olson & Kok (96) and Kok (59) observed that almost all of the abundant absorption changes in bacteria are partially reversed during a dark time between flashes of the order of 0.1 sec. A large band of increased absorption at 432 mμ (97), which had been ascribed

to the oxidation product of bacteriochlorophyll (94), restored much more slowly in the dark and invalidated this interpretation.

Reviewing the rather scanty observations of infrared absorption changes in bacteria, it seems that in respect to size, wavelength location, and sensitivity to extracellular conditions there is little analogy to the effects in aerobic photosynthesis. The kinetics of bacterial photosynthesis in general appear to need further elucidation.

Primary reductant.—Though not all workers agree on this point we feel justified in stating that all pigment catalysts so far observed in light minus dark spectra (carotenoids, cytochromes, chlorophylls) undergo oxidation upon illumination.

Surprisingly the primary reduced equivalents have not been observed either because of a very short life time or the absence of a color. Some spectroscopic observations that could indirectly shed light on this concern the reduction of pigment subtrates. Reduction of pyridine nucleotide in the light has been observed (55, 76) but only with slow techniques. Witt & Müller (71) made direct observations of the flash-induced changes of dye concentration in the Hill reaction. Detecting light was used of 570 mμ, which is absorbed by the oxidized form of DCPIP but not by the reduced dye (no chloroplast pigments show changes in this wavelength region). The changes were very small and the additive effect of a few flashes was required to quantitatively estimate the amount of dye reduced. It appeared that a flash of saturating intensity reduced two dye molecules (evolved one oxygen molecule) per some 12,000 chlorophyll molecules present, a yield some fivefold smaller than found for oxygen evolution in complete photosynthesis and with quinone reduction (77, 78).

A striking observation was made concerning the time course of dye reduction: it occurred instantly during the flash (10^{-5} sec.) but in the following dark time a partial ($\sim\frac{1}{2}$) reoxidation took place ($t\,\frac{1}{2}$ of the same order as that of the 475/515 change). If the chloroplasts were briefly heated to 50°C., reduction during the flash was unimpaired, but the back oxidation in darkness was complete so that no net reduction resulted. Whether these complex kinetics are restricted to the rather capriciously behaving DCPIP (cf. below) or have a general significance has yet to be determined.

Photosynthetic Pigments

Rodrigo (105) noticed a shift in the red absorption band of chlorophyll-*a* in concentrated solutions that he ascribed to an aggregation of the molecules. Brody (106) ascribed to the same phenomenon an anomalous fluorescence at long wavelengths that became particularly noticeable at liquid nitrogen temperatures. With intact *Chlorella* and *Porphyridium* cells a similar fluorescence was observed at low temperatures. Whether or not the two phenomena are identical, that is, chlorophyll aggregates exist *in vivo* or this fluorescence is due to another pigment with a longer wavelength absorption band than chlorophyll-*a*, is not known. The earlier observations that the fluorescence

yield of chlorophyll solutions declined with an exciting wavelength longer than 660 mμ have been shown to be due to the formation of non-fluorescent dimers [Lavorel (107), Weber & Teale (108)]. On the latter basis a correlation between the drop of quantum yield of photosynthesis and fluorescence in the far red does not appear likely. Thomas & Marsman (109) observed two red absorption maxima in *Porphyra* at 672 and 678 mμ. The absorption spectra and fluorescence yield of chlorophyll were unaffected by removal of phycobilins. Duysens (46) and Brody & Rabinowitch (110) suggested two types of chlorophyll complexes: one associated with accessory pigment, which is photosynthetically active and fluorescent, and a second type (the majority of pigment molecules), inactive in photosynthesis and nonfluorescent. Duysens' "inactive chlorophyll," in the light of present knowledge, is perhaps the form that requires concomitant excitation of accessory pigment. Brody & Brody (111) found that adaptation of algae to blue or green light changed the efficiency of phycoerythrin-sensitized chlorophyll fluorescence. This effect appears to be related to the wavelength adaptations in photosynthesis discussed by Emerson (1).

The different forms of chlorophyll-*a* found in plants have been discussed by French (112). In green cells the two most abundant pigments absorb at 673 and 684 mμ. A few pigments have been reported that, because of their long wavelength absorption and small concentration, appear likely candidates for the role of the final energy collector in photosynthesis. A 695 mμ absorption band has been found in *Euglena* cultures and a role in photosynthesis has been postulated for it by French (discussed elsewhere). Allen (113) observed that *Ochromonas danica* can exhibit considerable amounts of a pigment absorbing at 710 mμ. After extraction with organic solvents only chlorophyll-*a* is observed, therefore, as was long known in bacteria, different binding to protein probably causes the various absorption maxima. The pigment absorbing at 700 mμ, observed by Kok in difference spectra, has been purified to some extent (103). By extraction with acetone, 80 per cent of the chlorophyll could be removed without inactivation of the 700 mμ pigment, which in these preparations appeared to be equimolar with cytochrome-*f*. The parallel behavior of chlorophyll and P700 in extraction experiments and the observation of an absorption band at 430 mμ, behaving like the 700 band, supported the idea that the pigment moiety of the complex absorbing at 700 mμ was chlorophyll-*a*. Attempts to obtain a truly soluble preparation were not successful. It was possible to simulate the effect brought about by light (bleaching) by the addition of oxidizing agents. Agents that yielded chemical oxidation of P700 also stopped the light effect. Reducing agents, on the other hand, restored the absorption band at 700 mμ and at the same time the reversible photo-bleaching reaction. Attempts to determine the oxidation and reduction characteristics of the P700 complex showed that it behaved as a single electron transferring agent, characterized at pH 7 by an E_{h}' value of $\sim +0.43$ v.

These attempts towards characterization of P700 encouraged an inter-

pretation of the light-induced photochemical bleaching as an oxidation of the pigment with the concomitant reduction of an unknown reaction partner located on the particle.

Quantum Yield at Long Wavelengths and the "Enhancement Effect"

The decline of the quantum yield of photosynthesis at long wavelengths ($\lambda > 680$ mμ) and the synergistic effect of other wavelengths on far-red illumination were reviewed by Emerson (1) and by Spikes & Mayne (5). Since then several papers have appeared and will be discussed here.

Brody & Emerson (114) measured the wavelength dependence of the quantum yield at two temperatures with the red alga *Porphyridium cruentum*. The yield was poor in the blue, highest in the green and orange, and fell steeply at wavelengths longer than 644 mμ (probably the end of phycocyanin absorption). At the lower temperature the drop in quantum yield at long wavelengths was not so acute and, in the one point presented, could be additionally raised by supplemental light. Lowered temperature improved the quantum yield only in the far-red. Myers & French (115), however, using the electrode method, found the (light-limited) rate at 700 mμ to decrease more than the rate at 650 mμ when the temperature was lowered from 22° to 2°C. Emerson (116) also found that the quantum yield of far-red light could be improved by means other than supplemental light or lowered temperatures. Growing the algae in the presence of beef broth or earth extract extended the better quantum yields to longer wavelengths.

McLeod (117) found no enhancement in phosphate deficient cells. Addition of phosphate restored the enhancement within 24 hr. Addition of pyrophosphate reactivated the effect within 1 to 3 hr. and also stimulated it in normal cells. Further investigation of these factors appears highly desirable in view of the emphasis presently placed on enhancement data.

Action spectra.—Blinks (118), using his polarographic method, found that in the green algae *Chlorella* and *Enteromorpha*, the action spectrum for the enhancement effect was clearly that of chlorophyll-*b*, but had a shoulder on the long wavelength side of the red absorption peak that might be due to an involvement of part of the chlorophyll-*a* (λ maximum = 670 mμ).

Several algae containing phycobilins instead of chlorophyll-*b* were also investigated. In these organisms the action spectra for the enhancement generally followed the absorption of the respective phycobilin. Rabinowitch (119) published action spectra obtained by the late Robert Emerson. These correspond quite well with the estimated fraction of light absorbed by accessory pigment. A salient observation, not reported elsewhere, is that a "de-enhancement" or inhibition by supplemental light, occurs with certain wavelengths of added light (apparently those absorbed by chlorophyll-*a* and carotenoid). If an early saturation in light absorbed by the enhanced pigment is excluded, the explanation for this phenomenon is difficult. In this connection it is of interest that Blinks (118) observed equal enhancement

when chlorophyll-*a* was absorbing red or blue light, and concluded that the enhancement is an interaction of accessory pigment with chlorophyll-*a*. The same observation was reported by Haxo (120) with the red alga *Porphyra*. Myers & French (121), however, using *Chlorella* and a 647 mμ (chlorophyll-*b*) beam as the reference, and adding another beam of varied wavelength found enhancement only at wavelengths greater than 647 mμ.

French (112) believes the pigment responsible for the enhancement mediated by chlorophyll-*b* in green algae to be a form of chlorophyll-*a* absorbing at 695 mμ which Brown & French (122) found in *Euglena* and to a lesser extent in *Chlorella*. However, in the authors' opinion, this pigment, normally present in only a barely detectable amount, cannot be responsible by itself (i.e., without transfer to it from a more abundant pigment) for a measurable rate or enhancement. Furthermore—as a form of chlorophyll—it also should have an absorption band in the blue, capable of being "enhanced."

Interesting are the observations of enhancement in organisms without conspicuous amounts of accessory pigment but that are nevertheless capable of efficient photosynthesis. [It was shown by Allen (113) that green algae, which have lost their chlorophyll-*b* by mutation, are very poor photosynthesizers.] With *Botrydiopsis*, French (112) reported an action spectrum for enhancement of the rate at 700 mμ which peaked at 670 mμ. This was interpreted with the assumption that in this case a fraction of chlorophyll-*a* absorbing at this wavelength played the role of accessory pigment. The same phenomenon was reported by Govindjee & Rabinowitch (123) for the enhancement in the diatom *Navicula*. However, earlier observations of Emerson (119) with the same organism and apparently the same equipment showed fucoxanthol to be the almost exclusive sensitizer of enhancement and no 670 maximum. *Ochromonas danica*, which contains only chlorophyll-*a* and has rather poor photosynthetic capacity, did not reveal any enhancement.

Quantitative relations.—The maximum enhancement obtainable by mixing two light beams is of great interest. With *Chlorella*, Myers & French (121) found that saturation of the enhancement occurred when the rate produced by the 653 beam alone was twice that induced by the 700 mμ beam. If the increase caused by enhancement was attributed entirely to an improvement of the rate in the 700 mμ light alone, a maximum improvement by a factor of 1.8 was computed. If the enhancement was defined as the rate obtained with both beams simultaneously, divided by the sum of the rates produced independently, the maximal value was about 1.25. Blinks (118) reports slightly higher values for the maximum enhancement in a red algae. This may be because chlorophyll-*b* cannot be excited without also illuminating chlorophyll-*a*; whereas in red algae there is much less overlap of the absorption bands of the two involved pigments.

An action spectrum for photosynthesis in *Anacystis nidulans*, published by the authors (104), showed phycocyanin to be the main sensitizer and perhaps a small fraction of chlorophyll-*a*. Combining various ratios of 700 and 638 mμ light gave a maximal enhancement of the 700 mμ rate of about 1.7

times. Half-saturation of the effect occurred when the ratio of 630/700 rates was about unity. The published activation spectra of Emerson (119) also show a maximum enhancement of the 700 mμ rate by a factor of about 1.8 and one wonders whether special significance should be attributed to this value. However, the original curves of Emerson *et al.* (116) of the quantum yield as a function of (long) wavelength indicate much larger enhancement factors (virtually to infinity). This large discrepancy presents another barrier to a clear cut explanation of the enhancement. Possibly much could be gained if quantum yields rather than mere rates were determined. From this viewpoint it does not seem immaterial (112) whether one assumes that the 700 beam enhances the 650 beam or reversely. The quantum yield is known to be high at 650 mμ and low at 700 mμ, so that it seems preferable to accept that the poorly utilized light is assisted by the efficiently utilized. (Possibly more than one pathway of energy transfer is open to short wavelength light, whereas only one is open to long wavelength light.)

Time effects.—With the stationary electrode of Haxo & Blinks (124), Myers & French (115) observed a wavelength-dependent time course of photosynthesis. When light was given to chlorophyll-*a*, the rate of oxygen evolution initially rose very rapidly and then more slowly until a steady state was reached after roughly 6 min. When chlorophyll-*b* was illuminated, the steady state was reached more quickly (3 min.) and in addition a spike appeared very early in the rate curve. Upon termination of illumination a fast initial fall of the signal was followed by a slow decline to the dark level, taking as long as 3 min. to reach a nadir. With *Chlorella* the initial decay in the dark was found to be more rapid than the initial rise in the light and both were faster at 650 than at 700 mμ. At low temperatures (2°C.) the rates of rise and decay of the photosignal became very slow, 15 min. or more being required for the transitions. The rise at 650 mμ was still the more rapid one, although the decays were indistinguishable.

The transients in rates of photosynthesis, which occur upon switching from one illumination to another of equal effectivity but different wavelength, discovered by Blinks, have been investigated further by Blinks (118) and by Myers & French (115, 121). The latter authors report the transients to be a spike (increase in rate) going from sensitization by chlorophyll-*a* (or carotenoid) to that by chlorophyll-*b* and a depression in rate following a change of color in the opposite direction. Blinks observed slightly different transients in *Porphyra nereocystis:* when the light was changed from chlorophyll-*a* to phycoerythin, a sharp spike was followed by a depression and a gradual return to the steady-state rate. The transients were found to be only slightly temperature sensitive.

Action spectra for the transient produced when light was alternated from 700 mμ to a shorter wavelength are very similar to those obtained for the enhancement effect; both show that accessory pigments are involved. The transients were observed between chlorophyll-*b* and either the red or the blue (chlorophyll-*a*) absorption regions, both by Blinks and by Myers &

French. In contrast to the latter authors, Blinks (118) and Haxo & Fork (120) observed enhancement of the blue as well as the red chlorophyll band, and therefore complete parallelism between the two effects. Blinks suggests that the transients (and by implication also enhancement) may be caused by respiratory changes, i.e., the accessory pigment promotes an increased rate that lasts a short while after the light is changed.

The question of whether or not the enhancement effect occurs when the two beams are alternated in *time* was investigated with *Chlorella* by Myers & French (115). Lights of 650 and 700 mμ were alternated at equal intervals of 6, 1.2, or 0.6 sec. for each beam. The rate of O_2 evolution obtained in this manner was greater than that which either beam would have sustained if given continuously. The greatest stimulation was observed at the most rapid alternation (0.6 sec.); at this time interval, the enhancement was concluded to be equal to that obtained by giving 50 per cent of each beam simultaneously. This (together with the differences in decay of the rates produced by the two beams) suggests that the products of the two beams are stable intermediates, both of which are necessary for maximal rates of photosynthesis.

Myers & French also alternated two wavelengths (630 and 647 mμ) that did not give enhancement when presented simultaneously and found very slight, if any, enhancement. These two beams, however, did show transients when alternated slowly (minutes) but, curiously enough, not when alternation periods of 0.6 and 1.2 sec. were employed.

The present reviewers have some difficulty in judging the significance of the time-course data obtained with the stationary electrode method. No other technique with a known and fast enough time response revealed a measurable persistence of O_2 evolution after the light was turned off (69, 126, 127). Elsewhere in this review we discussed that similarly very little reducing power remains available after darkening. Some aspects of the electrode method, not a topic of this review, point to the desirability of independent confirmation of the time effects.

The enhancement effect has been shown to occur in the Hill reaction by Govindjee, Thomas & Rabinowitch (128). Oxygen evolution from *Chlorella* cells in the presence of quinone was measured. The quantum yield fell sharply at wavelengths longer than 680 mμ and could be raised by supplemental light of shorter wavelengths. The action spectrum for the supplemental light exhibited two peaks, one at 650 mμ and one at 670 mμ, with a very distinct valley in between. The authors point out that this analogy between photosynthesis and the Hill reaction suggests that the enhancement effect is neither attributable to an inhibition of respiration nor associated with the carbon dioxide reducing mechanism, but is in the oxygen evolution process common to photosynthesis and the Hill reaction.

Emerson also observed two similar maxima in the enhancement activation spectrum for *Chlorella* photosynthesis, but in his spectrum the 670 peak appears only as a weak shoulder on the 650 band. Neither Blinks nor Myers & French were able to resolve the two peaks. A valley between 653 and 670

is not observed in the absorption spectra of *Chlorella* cells. The data of Govindjee *et al.* thus suggest the existence of a pigment absorbing at 660 mμ that is incapable of sensitizing enhancement in the Hill reaction but capable of performing this function in photosynthesis. This would still further complicate the already confusing data concerning sensitization, enhancement, and transients.

In contrast to the decline in quantum yield and saturation rate at long wavelengths observed with photosynthesis and the Hill reaction, the rate of PMS-mediated photophosphorylation at high intensities is greatest at wavelengths beyond 680 mμ (104). Since this type of phosphorylation is not accompanied by oxygen evolution one could speculate, as was done in Figure 2, that oxygen evolution requires cooperation of two light steps. Possible support for this is found in the results of Petrack & Lipmann (129), showing that phycocyanin-free particles of blue-green algae, unable to carry out a Hill reaction, exhibited PMS-mediated phosphorylation.

Thomas & Govindjee (130, 131) studied photosynthesis in the red alga *Porphyridium cruentum*. For varying the ratio of light absorbed by chlorophyll-*a* and the accessory pigments, filters consisting of solutions of phycobilin pigments extracted from algae were inserted between an incandescent light source and the reaction vessel. As the absorption of the filter was in-

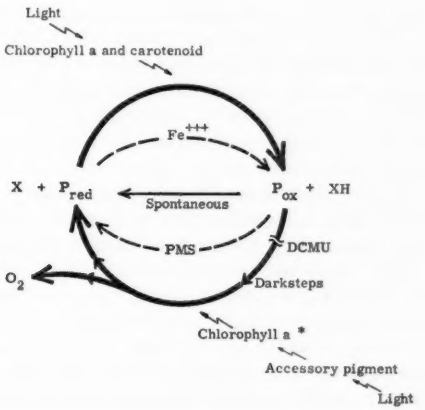

FIG. 2. An attempt to correlate observations concerning two photochemical steps in photosynthesis. One is sensitized by chlorophyll-*a* mainly, the other by accessory pigment associated with another smaller fraction of chlorophyll-*a*. The two steps oxidize and reduce P700, respectively. A spontaneous backreaction limits the lifetime of the photoproducts from the chlorophyll-*a* sensitized step. Phenazine methosulfate short-circuits the step driven by chlorophyll-*b*, whereas ferricyanide is thought to circumvent the chlorophyll-*a* step. The latter assumption definitely seems an over simplification. After Kok & Hoch (104).

creased from 0 to 20 per cent, the "relative quantum yield" did not change, from 20 to 80 per cent the yield declined linearly; and above 80 per cent absorption it fell steeply. At 95 per cent absorption of the filter, the rate of photosynthesis was negligible, even though the remaining 5 per cent of ($\lambda > 680$ mμ) light—if given as a monochromatic beam—would have yielded a detectable rate. A measurable rate was observed with the 95 per cent filter if an orange background light was used. The authors suggest that the inability to obtain a measurable quantum yield with the filter and white light may be due to an inhibition by extreme red light. Rabinowitch, Govindjee & Thomas (132) found photosynthesis in 700 mμ light to be 22 to 85 per cent inhibited by simultaneous illumination with 750 mμ light. One possible explanation of this inhibition is an effect of the light on respiration. Effects of red and far-red light on oxygen uptake of isolated mitochondria have been published recently by Gordon & Surrey (133).

McLeod (134) has applied the electrode method to measure the saturation rates of photosynthesis as a function of exciting wavelength. The rate at light saturation generally has been assumed to be limited by a "dark" reaction and therefore was thought to be independent of sensitizing wavelength. McLeod however, found definite structure in the spectrum. The *Chlorella* spectrum had peaks at 440 and at 650 mμ plus a shoulder at 680 to 694 mμ. The fall of the saturation rate at long wavelength has some suggestions of the quantum yield decline; however, addition of saturation intensities of 710 mμ to an already saturated 650 mμ photosynthesis gave no increase in rate. The spectrum for *Phormidium* shows a maximum in the region of accessory pigment absorption while *Botrydiopsis*, an alga containing only carotenoids as accessory pigments, has peaks at 420 and 680 mμ. McLeod suggests that the enhancement effect in some way is correlated with the structure exhibited by the saturation action spectra.

Govindjee *et al.* (135) studied the fluorescence of *Chlorella* cells excited by 700, 670, or 436 mμ light, or combinations of these. It was found that although at each wavelength the fluorescent intensity was strictly proportional to exciting intensity, combining 700 with 670 or 436 mμ light resulted in a fluorescence yield less than the sum produced by the individual beams. An explanation of these results consistent with enhancement data necessitates viewing fluorescence as an index of the fraction of the absorbed light not utilized in photosynthesis. If addition of shorter wavelength light makes excitation at 700 mμ more efficient, it would cause a drop in fluorescence yield.

Two Light Steps

The energy of several photons is required to liberate an oxygen molecule and much thought and speculation has gone into attempts to envisage such a multiquanta process (136). If, as seems likely, eight or more quanta are required per oxygen molecule evolved, this corresponds to two quanta per hydrogen transfer.

Evidence has accumulated which could indicate that these two quanta

are collected by different pigment groups and converted in different primary acts. Activation spectra of photosynthesis, fluorescence, and chemiluminescence (46, 124, 137) generally revealed a predominant role of accessory pigment compared to long wavelength chlorophyll. The enhancement effect in oxygen evolution, the sensitization of PMS-mediated photophosphorylation and of the bleaching and restoration of P700 can best be explained on the basis of two potentially useful sensitizations. The scheme, reproduced in Figure 2 was used by the present authors to visualize how a cyclic, two-quantum process driven by two pigment groups could explain the antagonistic action on the intermediate observed in difference spectroscopy and the synergistic effect in the overall production of oxygen. However, we should emphasize that presently no quantitative evidence is available proving that the two light steps are equally important, and one can speculate only on the mechanism by which the incident quanta are distributed optimally over the two pathways. For instance, French (112) correlated the enhancement data with the existence of two oxygen evolution reactions and three sensitizations, some of which are of minor importance. Franck has long been defending a theory in which a single chlorophyll molecule collects the energy of two quanta (138, 139, 140). The first excitation leads to the triplet state, the energy of which is stabilized in a complex with the primary acceptor, the second (a singlet excitation) dissociates the complex with the transfer of hydrogen. The mechanism which Franck envisages for the proper distribution of singlet and triplet states requires two forms of chlorophyll—one non-fluorescent (dry) and one fluorescent (wet).

Hill & Bendall (141) have presented a scheme involving two light reactions, in which the two cytochromes specifically associated with the chloroplast are functional. Cytochrome-*f* has an oxidizing potential of +0.365 v. and b_6 a potential of 0.00 v. In the first light step, water is split with the formation of a reduced moeity (primary reductant) and oxidized cytochrome-*f*. In a dark reaction cytochrome-*f* oxidizes cytochrome-b_6 regenerating reduced cytochrome-*f*. In the second light step cytochrome-b_6 becomes reduced and the oxygen precursor becomes oxidized. The precursor then dismutes, yielding molecular oxygen. As was discussed above, the actual evidence for a role of these cytochromes in the early photosynthetic events in chloroplasts has been rather negative so far.

Kautsky *et al.* (142) recently published high resolution time course analyses of fluorescence. From an analysis of data including intermittent illumination and inhibitor effects, the following conclusions were drawn. Two light reactions occur in photosynthesis, the first of which concerns an intermediate present in a concentration 1/400 that of chlorophyll. The product of the second light step is required to react with the product of the first step and regenerate its precursor. An oxidizing and a reducing equivalent are liberated in dark steps following the two respective light reactions. Not unlike the time courses of the 515 mμ pigment studied by Witt, *Chlorella* fluorescence followed a diphasic course; in the Hill reaction the second stage (indi-

cating the second light step) was absent. A rich field for contemplation opens up if one tries to correlate the two color effects with other two-light phenomena and with the time constants involved. The induction effects (66) and the requirement of preillumination for oxygen production by a single flash (143, 144) are both absent in the Hill reaction. The greater complexity of the kinetics of photosynthesis is apparent from many aspects—even from the difference between the respective light curves (cf. above). However, in the Hill reaction enhancement also has been observed between different wavelengths and possibly between successive flashes.

Chloroplast Reactions

Photooxidations.—Chloroplast-sensitized photooxidations have been recently investigated by Vernon and co-workers (157 to 160), Ikeda (161 to 165), and Nieman & Vennesland (166, 167). A question posed in discussions of photooxidation reactions is whether or not they are related to a normal physiological reaction (4). It appears however, that important information relating to normal physiological function (even if the reaction substrate is not itself physiological) can be obtained from studies of this nature. And, after all, photosynthesis comprises a photooxidation (of water) as well as a photoreduction (of CO_2). Vernon & Ihnen (157) interpreted their studies of the DCPIP-mediated oxidation of ascorbate by illuminated chloroplasts in the following way. Reduced DCPIP reacted with the photooxidant, which prevented the evolution of oxygen. Ascorbate in turn reduced the oxidized DCPIP. The photoreductant reduced a dye such as indigo carmine, FMN, or 1,2-naphthoquinone-4-sulfonic acid. In the presence of oxygen, reoxidation of the dye yielded the net result of photooxidation of ascorbic acid. Vernon & Hobbs (158) supported these deductions by spectrophotometrically measuring the reduction of indigo carmine and FMN resulting from anaerobic illumination of chloroplasts in the presence of DCPIP and ascorbate. A stoichiometry of about 1:1 was found between the amount of indigo carmine reduced and the amount of ascorbic acid oxidized. The reactions with FMN and indigo carmine were stimulated by a heat labile factor found in the chloroplast supernatant and had a broad pH optimum of pH 8 to 9.

Ikeda (161) found the rate of ascorbate-DCPIP oxidation stimulated by vitamin K_3 and FAD as well as by FMN. The rates with FMN and vitamin K_3 were very similar as long as a chloroplast supernatant fraction was added; without it, a better rate was obtained with K_3. Ikeda noticed the reaction was quite insensitive to inhibitors of oxygen evolution. Hydroxylamine, *o*-phenanthroline, azide, DNP, arsenite, and cyanide were more inhibitory to the FMN than to the K_3 system, which perhaps reflects different reoxidation pathways. [These inhibitor data remind one of Arnon's (168) results with the two cofactors in photosynthetic phosphorylation.] The rate of oxygen uptake increased linearly with light intensity and abruptly reached saturation. This type of response is reminiscent of the findings of Jagendorf & Avron (169) with PMS-mediated phosphorylation (in which O_2 evolution is also by-

passed). Addition of catalase plus ethanol approximately doubled the total oxygen uptake from a given amount of ascorbate, indicating that H_2O_2 was the result of cofactor reoxidation (however, it had only little effect on the rates).

Ikeda also studied the photooxidation of ascorbic acid in the absence of DCPIP (163). Compared to the DCPIP-mediated system the rate of oxygen uptake was much less and the plot of rate versus chlorophyll concentration showed no tendency to saturate at corresponding pigment concentrations used with DCPIP. The author attributed this to some natural mediator that was being added together with the chloroplasts. In the absence of DCPIP, the presence of an exogenous cofactor was essential for measurable rates of oxygen uptake. With menadione the pH optimum was 6.8 (7.8 for FMN). Here also, catalase-ethanol increased the final amount of oxygen consumed. That the absence of DCPIP greatly changes the system was shown by inhibitors. Oxygen evolution inhibitors (*o*-phenanthroline, hydroxylamine) now were quite effective at $10^{-3}M$, and the sensitivity to arsenite and PCMB increased. Addition of cytochrome-*f* or plastoquinone decreased the rate of O_2 uptake (164). Hinkson & Vernon (159) compared three photochemical activities of chloroplasts: the Hill reaction in which DCPIP is reduced and oxygen evolved, the DCPIP-mediated photooxidation of ascorbate in the presence of O_2, and the DCPIP-mediated photoreduction of indigo carmine with concommitant dehydrogenation of ascorbate. Heating chloroplasts at 51°C. for 10 min. and incubating at pH 8.5 for 30 min. inhibited the Hill reaction more severely than the other activities. Sonic oscillation, $10^{-3}M$ azide, incubation at pH 5.5 or incubation in water of low ion content preferentially suppressed the photoreduction of indigo carmine. $ZnSO_4$ at a concentration of $10^{-3}M$ preferentially inhibited the photooxidation. Treatment of the chloroplasts with digitonin drastically impaired their ability to carry out the Hill reaction and indigo carmine reduction but gave a sixfold increase in photooxidation rate. Use of 8-hydroxy-quinoline ($10^{-3}M$) and digestion with lipase or pancreatin did not affect photooxidation, but did affect the indigo carmine reduction and the Hill reaction almost equally. Phenylmercuric acetate at a concentration sufficient to completely inhibit both the Hill reaction and indigo carmine reduction caused only a 40 per cent reduction in photooxidation. Lysozyme and DNP had slight effect on any of the reactions.

Vernon & Zaugg (160) showed that the inhibition caused by DCMU, hydroxylamine, ammonium ions ($0.166M$), and aging of the chloroplasts could be overcome by adding DCPIP and ascorbate to Hill reaction systems employing TPN, DPN, FMN, indigo carmine, and vitamin K_3 as oxidants. However, addition of DCPIP and ascorbate to fresh and uninhibited chloroplasts decreased the rate of these reductions. An exception was observed with FMN. The rate of reduction in red light was stimulated almost twofold by the addition. Moreover, it was found that under these conditions the rate was considerably stimulated by hydroxylamine.

With TPN as the oxidant, addition of DCPIP and ascorbate to a fresh chloroplast preparation resulted in about 25 per cent inhibition of the rate of reduction. In this case three molecules of TPN were reduced per molecule of ascorbate oxidized. After the chloroplasts were aged for 45 hr. the rate of TPN reduction had decreased 90 per cent. Now addition of DCPIP-ascorbate restored the rate to the level of fresh chloroplasts plus DCPIP-ascorbate and one molecule of ascorbate was oxidized per molecule of TPN reduced. Still further aging resulted in a decreased rate of TPN reduction and the oxidation of more than one ascorbate per TPN reduced.

Margulies & Jagendorf (170) have confirmed and extended the work of Kumm & French (171) on the effects of dark storage of leaves and the light-induced reactions carried out by the chloroplasts isolated from them. The effects appear to be very similar to those of aging isolated chloroplasts. The Hill reaction and phosphorylation with FMN decrease rapidly with aging but not the ability to phosphorylate using PMS as a cofactor. The capability to reduce Hill oxidants could be restored by adding indophenol and ascorbate. In contrast to the isolated chloroplasts, aged leaves can be reactivated by light.

Thus it appears that the rate limiting step in aged chloroplasts or leaves is identical to the step that is inhibited in fresh ones by DCMU and hydroxylamine, and is generally identified as an O_2 evolving step. The experiments of Hill & Walker (178), which showed that with aging of chloroplasts the phosphorylation mediated by FMN and Vitamin K_3 (where O_2 is not evolved) decreased, support this assumption. Indeed, the protective effect of ascorbate during chloroplast aging, as noted by several authors, may well be through this sort of mechanism. The concept of comparative biochemistry is supported by these results, as they appear almost entirely analogous to those obtained in hydrogen adaptation of algae (7).

The photooxidation of cytochrome-*c* by digitonin-treated chloroplasts has been reported by Nieman & Vennesland (166). Reduced DCPIP was not photooxidized by such preparations. Intact chloroplasts show little net photooxidation of reduced cytochrome-*c*, presumably because the oxidized form can be reduced in a Hill reaction. [It was observed by San Pietro (175) that this reduction is greatly stimulated by the addition of PPNR.] Chloroplasts prepared by Nieman & Vennesland contained succinate-cytochrome-*c* reductase and cytrochrome oxidase activity (these enzymes are generally considered to be mitochondrial constituents). Nieman *et al.* (167) separated the cytochrome-*c* photooxidase activity into two heat-labile components. One contained chlorophyll and was insoluble in water except in the presence of detergent; the other fraction was water soluble. High concentrations of ammonium sulfate (0.4*M*) greatly stimulated the reaction.

Photophosphorylation and the Hill reaction.—Krogmann & Vennesland (172) have described a phosphorylation that they maintain occurs during the light-dependent oxidation of reduced indophenol dyes and have termed "oxidative photosynthetic phosphorylation." While chloroplasts will reduce

indophenol dyes at a very rapid rate, this reduction is accompanied by only a small amount of phosphorylation. Phosphorylation also occurs when indophenol is used in catalytic amounts, but the rate of phosphorylation is very low compared to that of other cofactors. It was found that under nitrogen up to 5 μM of dye could be reduced without any detectable uptake of inorganic phosphate. Thus, they argued that the phosphorylation assayed with catalytic amounts of dye occurred not during the reduction of the dye, but during its reoxidation. In the reviewer's opinion this interesting idea found only marginal support in the experiments designed to confirm it. In a similar system (ascorbate-indophenol) Jagendorf & Margulies (173) found that CMU completely inhibited the phosphorylation associated with the photooxidation but did not affect the rate of oxygen uptake. The authors suggested that the oxygen uptake in the light was not related to phosphorylation.

Krogmann (174) has extended the studies on the phosphorylation caused by illuminating chloroplasts in the presence of indophenol. The presence of this dye greatly stimulates the isotopic oxygen exchange catalyzed by illuminated chloroplasts (see below). Krogmann expected the oxidation of the photoreduced dye to result in hydrogen peroxide (Mehler reaction). Addition of a catalase-ethanol trap for hydrogen peroxide showed, however, the rate of hydrogen peroxide formation to be independent of the presence of indophenol. This would not seem to be consistent with a simple autooxidation of the reduced dye.

Nakamoto, Krogmann & Mayne (176) have provided isotopic data that establish the mode of action of several cofactors of "cyclic" phosphorylation. In a mass spectrometer experiment the production of oxygen (O^{16}) from a Hill reaction and the consumption (O^{18}) due to reoxidation of the Hill oxidant can be measured simultaneously. Therefore the rate of Hill activity can be determined although no net change in oxygen concentration occurs. The authors found that menadione, FMN, and trichlorophenol indophenol (in catalytic amounts) gave rise to an oxygen exchange reaction, indicating the reduced dye was oxidized by oxygen. If the reduced dye had been oxidized by the photooxidant, oxygen would have been produced and consumed. Thus, these reagents are probably "normal" Hill oxidants except that their reduced form is autooxidizable.

This work was confirmed and extended by Krall, Good & Mayne (177). For every oxygen atom (2 electrons) exchanged via FMN one molecule of phosphate was esterified. The P:O ratio for menadione exchange was considerably less. The authors suggest that quinoid oxidants in general are poorly coupled to phosphorylation (for example, phenolindophenol). In contrast, the exchange rate using PMS or pyocyanine was low but phosphorylation activity was high. In this case 5 to 15 molecules of phosphate were esterified for every atom of oxygen exchanged. The authors concluded PMS and pyocyanine catalyzed true "cyclic" phosphorylation; that is, their reduced forms are reoxidized by the photooxidant, while the reduced forms of FMN and menadione are not. The authors state "thus there is no longer any

reason for retaining the concept of 'cyclic' phosphorylation with FMN since molecular oxygen is certainly involved in the cycle." While phosphorylation with low concentrations of FMN had been previously shown to be oxygen-dependent, the isotopic method has not been applied to high concentrations of FMN that are independent of exogenous oxygen. Krall *et al.* give logical arguments why high concentrations would be seemingly oxygen independent; nevertheless, the experiments ought to be done. Additional evidence against a physiological "cyclic photophosphorylation" is provided by the study of Jagendorf & Margulies on CMU inhibition of a number of chloroplast reactions (173). The reactions fell into two groups on the basis of their sensitivity to CMU. A concentration of $3\times10^{-6}M$ CMU caused 50 per cent inhibition of Hill reactions with ferricyanide, indophenol, or TPN as oxidants and also the phosphorylations mediated by FMN, indigo carmine, or menadione. Phosphorylation was only inhibited 50 per cent at about $1\times10^{-3}M$ using pyocyanine or PMS if oxygen was rigorously excluded.

Assuming high concentrations of cofactors react the same way low concentrations do, there is apparently no physiological cofactor capable of running a true cycle in the presence of oxygen.

Hill & Walker (178) investigated photosynthetic phosphorylation employing PMS [Petrack & Lipmann (129) suggest the compound is chemically better defined as methyl phenazonium methosulfate] and pyocyanine. They concluded that the activity observed with PMS is due to its rapid conversion to pyocyanine in the light. Jagendorf & Margulies (173) confirmed the activity of pyocyanine but found PMS also active under conditions where its conversion to pyocyanine was prevented, although a higher concentration was required for maximal rates. The authors also confirmed that illumination of PMS in air led to its oxidation to pyocyanine. Illumination under anaerobic conditions apparently resulted in a reduction of the PMS by water (this has also been observed for FMN) as the absorption spectum was identical with that resulting from PMS and ascorbate.

Pyocyanine-mediated photosynthetic phosphorylation with fresh chloroplasts (whole or broken) was found to have a pH optimum of 7 with a shoulder at 7.7 by Hill & Walker (178). After 4 hr. aging, the broken chloroplasts showed only the pH 7.7 maxima. When FMN and menadione were employed as cofactors only one maximum at pH 7.7 was observed, either with intact or broken chloroplasts. Aging for 4 hr. reduced the activity with FMN and menadione to almost zero. Jagendorf (179) reported ferricyanide reduction by whole chloroplasts, in the absence of phosphorylating reagents, to have no distinct pH optima. Upon addition of Mg, ADP (phosphate buffer was used) a rather distinct optimum occurred at about pH 7.8. Uncoupling the chloroplasts by dilution resulted in a broad maximum suggestive of two peaks at pH 7.1 and 7.7.

Avron & Jagendorf (180) found the rate of ferricyanide reduction to be stimulated by arsenate only if ADP was present, whereas a measurable stimulation could be obtained with phosphate only. Arsenate was a competitive

inhibitor of phosphate in the phosphorylations associated with PMS and with ferricyanide reduction. The apparent K_m's for phosphate were 5.4 $\times 10^{-4} M$ with ferricyanide and $8.3 \times 10^{-4} M$ with PMS. The corresponding K_i's for arsenate were $6.7 \times 10^{-4} M$ and $14.2 \times 10^{-4} M$. No ATPase activity or ATP-inorganic phosphate exchange could be detected. The authors suggested a high energy ADP intermediate to explain the arsenate effect and lack of exchange. This is in contrast to oxidative phosphorylation systems where it is generally concluded a high energy inorganic phosphate intermediate exists. Later, however, Kahn & Jagendorf (181) presented evidence that indicated a high energy phosphate precursor in place of high energy ADP. Chloroplasts were incubated with inorganic phosphate in the light and then ADP added in a subsequent dark period. ATP was formed in the dark. The half life of the intermediate was 3 to 4 min. and the apparent concentration was one intermediate per about 500 chlorophylls. Kahn & Jagendorf (182) have also detected and purified an enzyme catalyzing C^{14}-ADP-ATP exchange. It appears that this enzyme is similar to that reported from mitochondria by Wadkins & Lehninger (183). Ammonium ions inhibit the enzyme at about the same concentration as they inhibit photosynthetic phosphorylation. The reaction rate of the enzyme is slower than overall phosphorylation but is of the same order of magnitude.

Arnon (184) has argued for more than one site of photosynthetic phosphorylation. He found a chloroplast extract to stimulate the phosphorylation rates in grana using FMN or vitamin K_3 as cofactors but not when PMS is employed. Arnon has also shown FMN and menadione to differ in their sensitivity to inhibitors and in their response to cofactor concentration (168, 184). Jagendorf & Avron (169) had found, and Arnon confirmed (184), that the rates of photosynthetic phosphorylation in weak light were better with FMN as cofactor rather than PMS. In strong light the opposite is true. Arnon reasons this may be interpreted as a "bypass" action of PMS. FMN and menadione may be obligately coupled to two sites of phosphorylation, one slow and one fast. PMS "bypasses" the slow site allowing greater rates in strong light. He reasons that the rates of the phosphorylation steps do not influence the overall rate in weak light, allowing a better rate with FMN because of its higher efficiency. *A priori*, one would then expect the ratio of rates with FMN and PMS in low light to reflect the number of phosphorylation sites involved in the two paths. Arnon's (184) data show the rate with vitamin K_3 to be 3.7 times the rate with PMS.

The phosphorylation system of chloroplasts appears to be more sensitive to photo-inactivation than does the electron transport (Hill reaction) system. Forti & Jagendorf (185) and Avron (186) have studied the inhibition of photosynthetic phosphorylation caused by pre-illumination of chloroplasts. The light inactivation could be partially prevented by cofactors of phosphorylation but this protection was negated if ADP was also added during the preillumination. Addition of phosphate or arsenate increased the protection afforded by the cofactor and overcame the harmful effects of ADP. Under

pre-illumination conditions where ATP formation was reduced to 50 per cent, the rate of TPN reduction was inhibited only 5 per cent.

Cell-free preparations of the blue-green alga *Anabena variabilis* that carry out photophosphorylation and photohydrolysis of ATP have been reported by Petrack & Lipmann (129). The small particles obtained by grinding the alga with alumina and extracting with 40 per cent ethylene glycol were essentially free of phycocyanin. Under anaerobic conditions, rates of phosphorylation of 200 to 400 mM/hr./mg. chlorophyll were obtained using PMS as the cofactor. When FMN or menadione were similarly employed the rates were lower and a combination of the two appeared to have a synergestic effect. Oxygen was quite inhibitory to the phosphorylation reaction and the inhibition was largely irreversible. The preparations were apparently incapable of catalyzing the Hill reaction. When the particles were prepared in 40 per cent dextrin, the phycocyanin was retained and phosphorylation occurred in the air, although at about one half the anaerobic rate. Thus the removal of phycocyanin appeared to be responsible for a photooxidation.

At low concentrations glutathione or cysteine stimulated the phosphorylation reaction; at higher concentrations they inhibited by inducing a light-dependent ATPase, which seemed to be closely related to phosphorylation. Both reactions required PMS and light and were inhibited by diiodobenzoic acid (an uncoupler of oxidative phosphorylation) and ammonium ions. The concentration of sulfhydryl compounds necessary to saturate the dephosphorylation rate was high, greater than $10^{-2}M$. The rates of dephosphorylation were faster when substrate amounts of ATP were replaced by a generating system. Photohydrolysis of ATP was also observed in spinach chloroplasts but the cysteine concentration had to be raised to 0.08 M.

The experimental finding that photoactive particles can be prepared either with or without accessory pigment should be of greatest importance in determining the role of accessory pigments in photosynthesis. On the basis of their results, Petrack & Lipmann (129) imply that phycocyanin is not essential for photosynthesis but functions as a protective agent against photooxidations. This conclusion does not seem to be justified since, as is well known, phycocyanin is a major sensitizer of photosynthesis in blue-green algae.

Avron *et al.* (187) and Krogmann & Jagendorf (188) have studied the light dependence of ferricyanide reduction as it is affected by simultaneous phosphorylation and "uncoupling" the Hill reaction from phosphorylation. The former authors found addition of magnesium and phosphate caused a large increase in the saturation rate but had little effect on the rate in weak light. More striking is the effect of "uncoupling" brought about by dilution. Here also a large increase in the saturation rate was observed, but in addition the low light rate was increased by 50 per cent. This is a very large increase and is certainly difficult to explain on the basis of one light reaction and all phosphorylations occurring in dark steps.

The "methemoglobin reducing factor" has been extensively purified by

Davenport & Hill (189). This enzyme stimulates the reduction of metmyoglobins and cytochromes and is, in fact, apparently the same enzyme as the photosynthetic pyridine nucleotide reductase purified by San Pietro and coworkers (175). Davenport & Hill found that approximately one-third of the whole leaf activity can be extracted from chloroplasts. The enzyme is sensitive to organic mercurials. No flavin, heme, or other prosthetic group has been detected (189). The purified enzyme will not reduce heme compounds with reduced pyridine nucleotides as electron donors. The function of this enzyme has yet to be elucidated.

The stimulation of the Hill reaction by carbon dioxide reported by Warburg & Krippahl (190) has been confirmed by Stern & Vennesland (191) and by Abeles, Brown & Mayne (192). Warburg interprets the effect of CO_2 as confirmation of his theory of photosynthesis; namely, the light reaction consists of a splitting of a bound CO_2 into O_2 and some reduced carbon form. Abeles *et al.*, using $C^{13}O_2$, could not demonstrate a cyclic evolution-uptake of CO_2. They also repeated the work of Boyle (193) purporting to show a CO_2 stimulation of the Hill reaction and concluded that Boyles' findings were the result of an experimental artifact. Although the explanation for CO_2 stimulation may not be as Warburg suggests, this finding will probably stimulate further investigations. Hill reaction velocity measurements were reported by Lumry *et al.* (203). The data were considered to support their earlier kinetic description of this process, which comprises a single light step k_l in the trapping center of the photosynthetic unit and a single, rate-determining dark step k_d regenerating this center. It was assumed that excited pigment chlorophyll molecules could either convey their quantum to the trapping center or—in case the latter was still occupied by a previous excitation ($t\ \frac{1}{2} \sim 10^{-2}$ sec.)—lose it as heat or fluorescence.

Reiske *et al.* (194) again observed the rate-intensity relations to follow this simple (Michaelis-Menten type) kinetics. However, an evaluation of the effect of light (substrate) attenuation across the reaction vessel revealed that intensity averaging, rather than the expected velocity averaging, gave a better fit for the macroscopic rate law. Also in view of other observations (125, 132, 188) it appears somewhat surprising that the Hill reaction should follow such simple kinetics. Reiske *et al.* found k_d to be temperature dependent with an activation energy of 13 kcal. The rate constant for the light reaction was essentially independent of temperature. Some variation of k_l with wavelength was found although the interference filters employed had so large a half-band width as to preclude much useful information on this score. In a footnote the authors state a quantum requirement of less than 7 quanta per oxygen molecule can be calculated.

Amesz & Duysens (195) measured the quantum yield of TPN reduction by spinach chloroplasts. They found an average value of 6 quanta per molecule reduced (or 12 per O_2) when the illumination was low intensity light of 688 mμ. When small concentrations of TPN were added to chloroplasts containing a large concentration of DPN, the reduction of pyridine nucleo-

tide was inhibited. The authors did not find any stimulation of pyridine nucleotide reduction by addition of ADP and inorganic phosphate. The only yields reported thus far for photophosphorylation were quite low (104). These data pertained to PMS mediation and were not specifically aimed at optimal values.

Levine (196) studied a mutant of *Chlamydomonas reinhardi* that was incapable of photosynthesis. Rate of growth in the dark on acetate, pigmentation, and chloroplast structure were comparable to that of the wild type. Whereas the rate of the Hill reaction with quinone as the oxidant was 73 per cent of the parent strain, the rate of CO_2 uptake and photophosphorylation (with FMN) were decreased about tenfold. Cell-free extracts of the mutant were capable of CO_2 fixation in the light when supplied with ATP. The author concluded that these data proved the requirement of photophosphorylation for CO_2 reduction. One could also conclude from the observations that the photosynthetically produced reductant is not available for ATP formation through oxidative phosphorylation (or that "dark generated" ATP is not available to assist CO_2 reduction).

Arnon has described an "electron flow theory of photosynthesis" (104, 197) in which a series of coupled oxido-reductions are driven by light via chlorophyll and are coupled with phosphorylation. Novel in this scheme is the splitting of hydroxyl ions by a cytochrome in a dark reaction. Cytochromes with the required potential (beyond +0.8 v.) however, are as yet unknown and *a priori* there seems to be no particular advantage in the splitting of hydroxyl ion (present in a concentration of $10^{-7}M$) over water ($55M$).

In bacterial photosynthesis the same author and also Stanier (198) digressed from van Niel's (199) classical description by assuming that the sole function of light in these organisms was (cyclic) phosphorylation. In the case of purple non-sulfur bacteria supplied with organic hydrogen donors, it is certainly feasible that phosphorylation represents a sizeable fraction of the light energy conversion. The same could still hold in the case of green and purple sulfur bacteria utilizing a strong reductant such as molecular hydrogen. If grown on weak reductants it still could be possible that energy from light-generated ATP drives a reduction of pyridine nucleotide (for instance such a phenomenon must occur in nitrite oxidizing autotrophs). Evidence against the sole formation of ATP is, first, the constancy of quantum yield observed in sulfur bacteria regardless of the strength of the exogenous reductant and, second, the absence of discrimination between such different reductants (200). A more complete discussion of bacterial photosynthesis was given by Frenkel (201). Recently, Losada, Nozaki & Arnon (202) succeeded in obtaining the production of molecular hydrogen from thiosulfate in the light with intact *Chromatium* cells. This important observation necessitated a return to van Niel's concept. It was now assumed that light generated a strong enough reductant (excited chlorophyll) to reduce protons to H_2 whereas the remaining oxidant is removed by the external hydrogen donor (via cytochromes).

We would like to point out here that whereas almost all conceivable substrates and cofactors have been thoroughly investigated with chloroplasts, the "concentration" of the most important substrate—light—has received rather scanty attention. The relatively simple measurement of rates as a function of intensity can be quite informative. In the literature of chloroplast reactions two different types of such curves are met. One follows the familiar Michaelis-Menten type kinetics if intensity is plotted as substrate concentration. In this case the reaction rate appears to be determined only by one light and one dark reaction. Examples of this are the Hill reaction (194) and photophosphorylations mediated by vitamin K_3 and FMN (184). The second type of intensity curve ascends more linearly in weak and medium light and reaches a saturation level more abruptly. In this case a more complex—as yet unexplained—mechanism must prevail. This type of kinetics is exhibited by complete photosynthesis (95) and, as it seems from the few data available (161, 169), even more pronounced by photooxidation of DCPIP-ascorbate and PMS-mediated phosphorylation.

Primary Reactions

The crucial phenomenon in photosynthesis, of course, is the first step(s) in which the energy of captured photons is stabilized in chemical potential. Such a primary event should occur with a high quantum yield and low temperature coefficient. As long as we consider absorption and transfer of photons within the pigment arrays we deal with life-times of the order of 10^{-9} sec. (145) [or some 10^{-3} sec. for the longer lived triplet state (45)]. Clearly these times are too short to visualize efficient coupling with ordinary (temperature dependent) collision type chemical reactions. About the only way one can visualize the primary light reaction is that it results in the separation of two entities [e.g., (H) and (OH) or "electrons" and "holes" to use some common phrases] with a sufficiently large activation barrier between them to prevent an immediate backreaction, i.e., to yield a long enough life-time for the products so that they can be processed further. One cannot visualize such a course of events to occur in a single small molecule, but rather has to conceive an ordered multicomponent structure where the reaction partners are prearranged in a favorable spatial distribution.

A second phenomenon, which can only be explained on the basis of a cooperation (at least a close spacing) of several molecules, is the photosynthetic unit. Though there is only one stabilization center per 400 chlorophylls, the high efficiency of photosynthesis shows that regardless of which pigment molecule absorbed it each photon has a high probability of reaching this trap.

The mode of energy transfer within the unit is still under discussion (146 to 149). Two mechanisms have been considered, resonance and charge transfer, and were lucidly discussed by Rabinowitch (150). Fluorescence sensitization studies have adequately proven that resonance transfer does occur in the pigment arrays. However, fluorescence is a loss process, restricted to a few per cent of the absorbed quanta and only indirect evidence supports

this mechanism as being adequate to provide for the bulk of the energy transfer within the unit. Nevertheless, this concept presently seems the more likely since data brought forward to support the charge transfer mechanism appear even less quantitative and direct.

An advantage ascribed to the solid-state model is its mode of separating the primary products. However, it is entirely feasible to conceive a mechanism occurring on a single particle where a light quantum induces the transfer of a hydrogen or electron from one to another active group on the surface with a simultaneous deformation (e.g., uncoiling) of the protein, which now presents a barrier to the backreaction. Therefore a discussion of the two mechanisms appears academic if extended to the trapping centers.

A by now well known example of such an "isolated" photochemical event occurring in a chloroplast pigment complex (holochrome) is the photochemical transformation of protochlorophyll to chlorophyll, studied by Smith and co-workers (151). The transfer of two hydrogens to protochlorophyll from an unknown donor (152) occurs in the complex with a quantum yield close to one per hydrogen, and takes place even at −80°C. (but not at liquid nitrogen temperature).

Recently, Arnold & Clayton (75) published another series of data which they interpret as supporting the "electronic" nature of the primary events in photosynthesis. Films of dried chromatophores of *Rhodopseudomonas spheroides* showed a high yield of delayed light emission compared to living cells, and a significant photoconductivity sensitized by bacteriochlorophyll. Upon illumination a sudden change in dielectric constant—indicating separation of charges—was observed. Spectral changes induced by illumination were also studied with whole cells, chromatophores, or films of dried extracts of *R. spheroides*. The light minus dark spectra of dried chromatophores from normal cells varied very little between 300° and 1°K. and were interpreted as showing a slight shift towards shorter wavelength of all absorption bands. This general shift was ascribed to a change of the ground state caused by the electronic field generated by the separated charges. Samples briefly exposed to methanol vapors (which presumably disrupted the carotenoid from the structure) or prepared from a carotenoid-free mutant showed no shifts of carotenoid bands. At 1° and 77°K. a second order decay of the shifts was observed upon darkening; this was interpreted as a recombination of electrons and holes. At room temperature, and more so at 5°C., return of the change after illumination was retarded. This was ascribed to the trapping of electrons occurring at the higher temperature only. In whole cells of the mutant strain no light-induced absorption changes were seen (at 27°C.) unless photosynthesis was increased with increasing degree of inhibition. This observation was thought to indicate that in non-inhibited cells no primary electrons accumulate because of their removal in subsequent photosynthetic reactions. This argument seems to be somewhat complicated by the presence of the light-induced changes in active whole cells of the normal

strain—in accord with the evidence of other workers that is discussed elsewhere in this review. We should recall that Smith & Ramirez (153) observed a bandshift of the carotenoids towards longer wavelength and that the 890 band seemed to diminish rather than shift.

Chance & Nishimura (154) observed that the light-induced (and bacteriochlorophyll-sensitized) oxidation of cytochrome 423.5 mμ occurred in *Chromatium* (but not in other photosynthetic bacteria) to a substantial extent even at 80°K. with the same rate and quantum yield as at room temperature. High efficiency was assumed for this conversion since it was earlier shown in *R. rubrum* to require two quanta per electron transfer (155). The fact that at low temperature only a single cytochrome was oxidized via bacteriochlorophyll (at room temperature several are involved), was considered in accord with earlier observations concerning energy transfer between cytochromes in the respiratory chain which, by its temperature dependency, seems to involve collision processes (156). Chance & Nishimura assumed that upon excitation by light, bacteriochlorophyll accepts an electron or a proton from the cytochrome and becomes reduced. However, the data of Duysens and Goedheer (discussed previously), duplicated at low temperatures by Arnold & Clayton (75), indicate that bacteriochlorophyll becomes oxidized in light. This would require acceptance of a third moiety in the chain of events that becomes reduced in light but escapes spectroscopic detection. The latter interpretation would also be in better analogy with the photobleaching of chlorophyll 700 in aerobic photosynthesis, which in all likelihood is also a primary event.

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PHOSPHORYLATION IN HIGHER PLANTS[1,2]

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Introduction

In the economy of life processes, phosphorylation occupies a place comparable to that of nucleic acids in protein synthesis and heredity transmission, of proteins as specific catalysts, and of oxidation-reduction systems in energy transfer.

Phosphorylation may be defined as the biochemical process by which phosphate or phosphoryl radicals are transported to an acceptor by a transfer reaction. As a consequence of phosphorylation the reactivity of a compound increases. This is due, in many cases, to the capacity for substituting the phosphoryl group with other groups or radicals (synthetic reactions); or, in other cases, to a higher capacity for binding to active constituents, for instance enzymatic proteins, or for forming complexes with metal ions.

As a result of phosphorylation, activation energy barriers are lowered and otherwise unfavorable thermodynamic conditions are overcome. The number of reactions chemically feasible in biological systems thus is enormously increased.

Our knowledge of phosphorylation in higher plants has been built up mainly through studies aimed at elucidating mechanisms already well known and thoroughly investigated in animal cells and in microorganisms. All available evidence indicates a fundamental identity for the process in all living organisms.

The main purpose of the present article is to present a general view of phosphorylation in higher plants. Particular attention will be paid to the physiological aspects and implications of this process. The importance of phosphorylating reactions for the control of such functions as respiration, growth, and solute uptake *in vivo* will thus be treated in some detail. Recent evidence concerning the regulation of phosphate metabolism in intact cells will also be briefly discussed.

The reference list reported in this article is by no means complete. This is at least in part justified by the existence of a large number of recent reviews or general papers dealing with this or closely related subjects. Among these are: Atkinson & Morton (1), and Pullmann & Pullmann (2) on the general mechanism of phosphorylation and the energetics of phosphate bonds; Kalckar (3), Leloir *et al.* (4), and Hassid *et al.* (5), on the role of phosphory-

[1] The survey of literature pertaining to this review was completed in September 1960.

[2] The following abbreviations will be used: DNP (2,4-dinitrophenol); Ptp (phosphate, or phosphoryl, transfer potential); UDPG (uridine diphosphoglucose); UTP, UDP, UMP (uridine tri- di- and monophosphate).

lated intermediates in the interconversion of sugars; Lehninger (6) on oxidative phosphorylation; Kornberg (7) on the synthetic role of pyrophosphorylasic reactions; and Kornberg (8) and Heppel & Rabinowitz (9) on the involvement of phosphorylating reactions in the synthesis of nucleic acids. Useful information on phosphate metabolism is also found in articles dealing with respiratory metabolism [Axelrod & Beevers (10), Hackett (11), Gibbs (12), Laties (13)], metabolism of lipids [Stumpf (14)], and amino acids [Webster (15), Yemm & Folkes (16)].

The central point of oxidative phosphorylation at the mitochondrial level will be considered extensively in this *Review* by Crane, and is thus only superficially treated in the present article. Photosynthetic phosphorylation has also been recently reviewed (17, 18) and is not considered in this article.

Methods for the Study of Phosphate Metabolism

While many of the classical procedures for the determination and fractionation of phosphorylated compounds are still valid and widely used, a few recently developed procedures seem worth mentioning here, either because of their specificity and convenience, or because they permit us to overcome some technical difficulties characteristic of plant materials [cf. Albaum (19)].

A convenient adaptation of the Fiske and SubbaRow method to the rapid determination of very small amounts of phosphate in cell-free systems has been elaborated by Taussky & Shorr (20). Rapid and quantitative separation of organic from inorganic phosphate has been obtained with the isobutanol-benzene method by Lindberg & Ernster (21); it may be useful to observe that the presence of reducing agents such as ascorbate interferes with the procedure. This inconvenience is eliminated by the omission of benzene, as in the original Berenblum & Chain method (22). The integration of this method with the use of radioactive phosphate is very convenient for the detection of low rates of phosphorylation in cell-free systems. Inorganic phosphate, hexose phosphates, and adenosine phosphates can be efficiently determined in cell-free systems through a procedure combining paper chromatography and the use of radioactive phosphate [Lindberg & Ernster (23)]. Good results in the separation by column chromatography of phosphate intermediates in extracts from whole cells are reported by Ducet & Mencl (24), who used a modification of the Khim & Conn method (25), and by Bartlett (26). Paper chromatography has been widely used for the determination and identification of ^{32}P- and ^{14}C-labeled intermediates in higher plant tissues [Rappaport & Chen (27), Loughman & Martin (28)].

All of these methods, though giving excellent results with cell-free, partially purified systems, are often unsatisfactory for the quantitative determination of phosphate compounds in extracts from higher plant tissues, in which various substances may interfere with the clear-cut separation of the compounds investigated. Thus, the classical method of fractionation with barium and alcohol fails, in many cases, to give reproducible and quantitative results. On the other hand, the presence in plants of phosphate-contain-

ing materials still unknown or unsufficiently characterized may cause significant errors. A convenient approach to this technical problem is the combination of a preliminary, partial fractionation, with the enzymatic measurement of the various phosphate metabolites by means of specific enzymes. This type of procedure has been successfully employed for ATP determination by Marré & Forti (29), who combined nucleotide adsorption on Norit A charcoal [Crane & Lipmann (30), Forti (31)] with the use of rabbit muscle ATPase. A modification of the enzymatic method of Slater has been elaborated by Rowan (32) for the determination of hexose phosphate, ATP, and ADP. Further development of these quantitative and specific techniques appears highly desirable.

Level of Phosphorylated Intermediates in Higher Plants

The presence in higher plants of most of the phosphate compounds that occur in animal tissues and in lower organisms is well documented. However, relatively few data are available concerning the average concentration of the main phosphorylated constituents and their changes in relation to various physiological situations. A survey of recent reports in this field indicates that hexose phosphate and adenosine phosphate, together with inorganic phosphate, are probably the quantitatively more important and widespread components of the acid soluble fraction. In Table I some data on hexose phosphate, ADP, and ATP concentrations in higher plant tissues are reported. It appears that hexosemonophosphates predominate among sugar esters, their concentration ranging between 1 and 6 μM/gm fresh weight, and the equilibrium between glucose-6-phosphate and fructose-6-phosphate being near enough to that found *in vitro* for the phosphohexoseisomerase reaction. Glucose-1-phosphate and fructose-1,6-diphosphate concentrations are significantly lower, ranging between 0.3 and 2 μM/gm fresh weight. A consideration of the equilibrium constants of the reactions involved in the interconversion of the hexose esters suggests that the activity of phosphofructokinase—besides, obviously, the availability of ATP—may be a factor of major importance in the control of this important point of carbohydrate metabolism [cf. Wu (37) for the analogous situation in animal cells].

According to the few data available, ATP and ADP concentrations vary around 0.2 to 1 μM/gm fresh weight for each of these compounds. A qualitative evaluation by Cherry & Hageman (38) of other nucleotide polyphosphates in pea tissues shows that guanosine triphosphate and uridine triphosphate (UTP) are present at a much lower concentration. The same report also indicates the presence in this material of small amounts of a guanosine-adenine dinucleotide, which could possibly be related to Albaum's report of a polymerized form of ATP in plant tissues (19).

The scarcity of investigations in this field makes it difficult to draw any general picture correlating the changes in concentration of the above mentioned major phosphorylated metabolites with known physiological activities. In tomato ovaries (33), as in pea seeds (34), the initial stages of development and the initiation of protein and starch synthesis correspond to a

TABLE I

AVERAGE LEVEL OF ACID-INSOLUBLE PHOSPHATES IN SOME HIGHER PLANT TISSUES

Material	μM/gm fresh weight*							
	Total acid-soluble phosphate	Inorganic phosphate	Glucose-1-phosphate	Glucose-6-phosphate	Fructose-6-phosphate	Fructose-1,6-diphosphate	ATP	ADP
Pollinated or parthenocarpic tomato ovaries (2 to 6 days from pollination) (33)	15–19	10–12	0.6–0.9	2.1–2.9	0.8–0.9	1.9 –2.2		
Developing pea seeds (12 to 21 days from anthesis) (34)	17	9	1–6			0.15–0.3	0.3–0.8	0.2–0.5
Mature pea seeds (imbibed overnight, aerobiosis) (35)		2	1–9			0.4 –1	0.5–1	0.4–2
Etiolated pea internode segments (with $5\times10^{-6}M$ IAA) (29, 36)		6		0.5			0.2–0.3	0.3–0.4

* Data roughly approximated from calculation of values reported in the original papers.

significant increase of hexosephosphate and to a decrease of inorganic phosphate, while the ATP/ADP ratio, in the single case where it has been determined, does not show significant changes. In tomato and pea fruits the intense starch synthesis is accompanied by a progressive, strong increase of phosphorylase activity (39); on the other hand, the glucose-1-phosphate/inorganic phosphate ratio is such that the reaction catalyzed by this enzyme should follow the phosphorolytic direction.

One has to consider the difficulty of interpreting these data without a knowledge of the real concentration of reagents at the level of reaction sites. In this connection, Rowan & Turner (34) suggested that the lack of correlation between starch synthesis and the glucose-1-phosphate/phosphate ratio might be due to a large difference between the apparent ratio and the one prevailing at the cell site where starch is synthesized. It is interesting to quote, in this regard, that Wu and Racker suggest that in isolated ascites tumor cells the active concentration of inorganic phosphate available for the triose phosphate dehydrogenase reaction is probably considerably lower than that appearing from the analysis of the cell extracts (40). An analogous suggestion of the presence of separate "stores" of ATP and ADP in intact ascites tumor cells has been advanced recently by Chance & Hess (41).

The few available data on ATP, ADP, and "high energy" phosphate concentrations are of obvious interest. Their significance in relation to the problem of control of metabolism will be discussed in a further section. Among other important phosphorylated compounds, the presence in higher plants of almost all phosphorylated coenzymes and cofactors has been qualitatively demonstrated [see reviews on respiration (10 to 13)].

Quantitative data are available for pyridine nucleotides; Anderson & Vennesland (42) found in green leaves of several species a concentration of DPN ranging between 0.01 and 0.02 μmoles/gm fresh weight and an approximately equivalent amount of TPN. Quite recently, Krogmann reported values of 4 μmoles/gm chlorophyll for DPN and TPN (not distinguished) in chloroplasts (43). According to Bevilacqua (44, 45), the concentration of total pyridine nucleotide coenzymes during the first week of germination varies from about 2 to $5\times10^{-5}M$, for wheat embryo, and from 1 to $3\times10^{-5}M$, for pea embryos. Enzymatic determination of TPN in isolated, etiolated pea internodes gave values of 1.1×10^{-5} and $0.9\times10^{-5}M$, for reduced and oxidized TPN (46) respectively. The ratio between the reduced and the oxidized form was consistently shifted towards reduction by the presence of indoleacetic acid in the medium.

Phosphate Turnover

In higher plants, as well as in all other organisms, phosphate turnover comprises three distinct phases. In a first phase, inorganic phosphate is taken up and combined with organic molecules or radicals, forming amide, ester, or anhydride bonds (phosphorylation *sensu strictu*). From the primarily phosphorylated compound thus formed, the phosphoryl group may be trans-

TABLE II

FREE ENERGY CHANGES OF HYDROLYSIS OF SOME PHOSPHORYLATED METABOLITES

Metabolite	Temperature (°C.)	pH	Free energy change in kcal. at specified temperature and pH
ATP (→ADP + inorganic phosphate)	30	7	− 7.0
ATP (→AMP + pyrophosphate)	37	7.5	− 8.6
ADP (→AMP + inorganic phosphate)	25	7.0	− 6.4
Pyrophosphate	30	7.0	− 6.5
Glucose-1-phosphate	30	7.0	− 5.0
Galactose-1-phosphate	25	7.0	− 5.0
Glucose-6-phosphate	25	7.0	− 3.3
Fructose-6-phosphate	30	7.0	− 3.8
Fructose-1-phosphate	38	5.8	− 3.5
Glycerol-phosphate	25	7	− 2.1
Glyceraldehyde-1,3-diphosphate	25	6.9	−11.8
Phosphoenolpyruvate	30	7.4	−12.7
Acetylphosphate	29	7.0	−10.1

[Data selected from Atkinson & Morton (1).]

ported to other molecules (transphosphorylation). Finally, phosphate—or pyrophosphate—is split from the phosphorylated intermediates either through hydrolytic cleavage, or through its substitution by an organic radical.

The phosphate bonds of biologically interesting compounds are usually classified, since the classical work of Lipmann, as high- and low-energy bonds on the basis of the free energy change of hydrolysis. However, the transition between the two categories is rather continuous. For this, and for other general considerations the proposal by Klotz (47) to indicate the "energy wealth" of phosphate bonds as the Phosphoryl transfer potential (Ptp) seems to be accepted, and this term will be adopted in the present article in many instances. The value of the Ptp, expressed in kcal., corresponds to the amount of biochemical work that can be obtained from a given phosphate bond.

Table II reports some data on the free energy change of hydrolysis (equivalent to the Ptp) of several important phosphorylated metabolites. Among these, the correction should be noted for ATP (terminal bond) from the higher early value of 10 to 12 kcal. to one of about 7 kcal.

In a study of the electronic structure of the phosphate bond Pullman and Pullman (2) have recently calculated the theoretical contribution of different factors to the Ptp of several energy-rich phosphorylated compounds. According to these calculations, opposing resonance and electrostatic repulsion between the positively charged phosphate atoms for ATP and ADP, opposing resonance and free energy of ionization for acyl phosphates, and opposing

resonance and enol-keto tautomerism for phospho*enol*pyruvate, appear to be the main factors in the very high free energy change of hydrolysis of these phosphate compounds.

Primary Phosphorylation

The mechanism of phosphate incorporation into organic compounds appears, at least in the cases most extensively investigated, substantially similar to that found in animal cells. The main source of energy for this endergonic process is the redox potential energy that is set free in oxidative metabolism, even if some minor contribution may come from the bond energy (group transfer potential) of compounds attacked by phosphate in phosphorolytic reactions, as in the case of phosphorolysis of glucosidic linkages.

A problem still open is whether phosphorolytic cleavage of starch or other polysaccharides significantly contributes to phosphorylation in higher plants, by saving, as in animal tissues, a large part of the energy of the glucosidic 1–4 bond and thus sparing one of the two molecules of ATP required to raise the free hexoses to the level of fructose diphosphate. Starch phosphorylase is a widespread enzyme in plant tissues; moreover, as stated in an above section, the apparent glucose-1-phosphate/inorganic phosphate ratio in plant tissues is much in favor of starch breakdown. However, all available evidence seems to indicate that maximal phosphorylase activity in a tissue accompanies the stages of synthesis, while starch breakdown is concomitant with an increase in amylase activity [Marré (48, 49)]. It seems possible that two different pathways are involved in starch breakdown: one, hydrolytic, used for rapid, massive production of soluble sugars to be transported to other cells; and another, quantitatively minor, leading to the phosphorolytic synthesis of hexosemonophosphates for respiration or anabolic purposes.

The occurrence of a phosphorylating step at the level of glyceraldehyde-3-phosphate oxidation is well established, and the respective enzymes (glyceraldehyde-3-phosphate dehydrogenase and phosphoglyceric acid kinase) have been partially purified and characterized (50, 51). While the glyceraldehyde-3-phosphate dehydrogenase of non-photosynthetic tissues is DPN dependent and very similar to the animal and yeast enzyme, two additional dehydrogenases acting on the same substrate are present in green tissues. One of these is TPN specific and phosphate or arsenate dependent, and the second is also TPN specific but is active even in the absence of arsenate or phosphate (52, 53), and thus apparently is uncoupled from the normal phosphorylating reaction. It has been suggested that TPN-linked triosephosphate dehydrogenase may be important for photosynthetic metabolism,

A large body of evidence indicates that in higher plants, as in animal tissues, the bulk of phosphorylation occurs at the mitochondrial level during the stepwise transfer of electrons from respiratory substrates to oxygen. The mechanism of oxidative phosphorylation being discussed in another chapter of this volume, only a few general points of this process will be touched here. Phosphorylation coupled to oxidation of the tricarboxylic acid cycle sub-

strates has been demonstrated to occur in the mitochondria of a large number of species (11, 13). Isotope studies show that the Krebs cycle is usually the major route of oxidative carbohydrate breakdown (10, 12). An electron transfer chain involving pyridine nucleotide coenzymes, flavoproteins, cytochromes-*b* and -*c*, and cytochrome oxidase as the terminal oxidase appears to be the most important system involved in mitochondrial oxidative phosphorylation, and, even in whole plant cells, modern work tends to assign a predominant role to cytochrome oxidase in mediating overall oxygen uptake (11, 54). Uncoupling agents such as 2,4-DNP affect mitochondria of higher plants in the same way as they do those of animals, by completely uncoupling oxidations at the electron transport chain level from phosphorylation, while they do not affect phosphorylation at the substrate level (11).

On the other hand, the possibility is still to be considered that other respiratory pathways besides the Krebs cycle might bring on, in higher plants, phosphorylation coupled to oxidations. This possibility appears of particular interest in the case of the pentose phosphate pathway, which recent researches indicate as one of the main respiratory routes, at least under particular physiological conditions (12, 55, 56). During the oxidation of glucose-6-phosphate to pentose, two TPN molecules are reduced. A problem arises then of the system mediating the oxidation of this TPNH, and its possible role in phosphorylation. This point is still largely obscure; however, some data are available that can be useful at least to start its discussion.

Investigations *in vitro* show the presence, in higher plant tissues, of at least five distinct systems which could mediate electron transfer from TPNH to oxygen:

(*a*) TPNH + pyruvate + CO_2 (malic enzyme) → malate → mitochondrial oxidations (57)
(*b*) TPNH + α-ketoglutarate + CO_2 → citrate (11)
(*c*) TPNH → glutathione → ascorbate/dehydroascorbate → ascorbate oxidase (58)
(*d*) TPNH → glutathione → ascorbate → quinones → polyphenoloxidase (58)
(*e*) TPNH → ascorbate/monodehydroascorbate → ascorbate oxidase (59), or quinones-polyphenoloxidase, or cytochromes (60).

The first of these pathways appears as a mixed system, in which the pentose phosphate pathway would provide the primary electron donors and the initial electron carrier; then electrons from TPNH, pyruvate from glycolysis, and CO_2 would combine to form malate, a substrate for the mitochondrial oxidations of the Krebs cycle. In the step from malate to oxalacetate, three phosphorylating steps would occur, obviously, even if undirectly, at the expense of the oxidation-reduction energy primarily stored in TPNH by pentose phosphate pathway oxidations. Evidences in favor of the operation of this pathway under physiological conditions are: first, the presence of an active TPNH specific malic enzyme in the soluble fraction of higher plants (57), and second, the demonstrated capacity of plant tissues to incorporate labeled CO_2 rapidly into malate (61). One must also consider that a large part of the carbon fragments entering the Krebs cycle is used on the way towards oxalacetate for anabolic purposes, so oxalacetate has to be continuously regenerated in order to maintain a constant efficiency of the

cycle. The synthesis of malate from TPNH, CO_2, and pyruvate could be an active system for the maintenance of a steady oxalacetate level in mitochondria. The quantitative importance of such a system should probably be greater when large amounts of organic acids are removed from the Krebs cycle, for instance, during active synthesis of proteins. On the other hand the presence of other CO_2-fixing, dicarboxylic acid-producing mechanisms must be remembered. In fact, the phospho*enol*pyruvate carboxylase system appears as perhaps the most active mechanism implicated in the CO_2 incorporation into malate in higher plants (12).

For the pathway involving glutathione and ascorbic acid, no considerable difficulty is met so far as hydrogen transfer from TPNH to ascorbate is concerned, as the enzymes involved have been shown to be of wide occurrence in higher plants (58) and the occurrence of the reaction between glutathione and dehydroascorbic acid has been demonstrated *in vivo* (62). The doubtful step in this sequence concerns rather the efficiency of both ascorbic acid oxidase and polyphenoloxidase as terminal oxidases in whole cells. Eichenberger & Thimann (63) found that in pea stem tissue only a few per cent of total respiration can be mediated by ascorbic oxidase although this enzyme shows very high activity in extracts from this material. In aged potato slices, where the operation of the pentose phosphate pathway appears very active both from enzymatic (64) and from isotope measurements [ApRees & Beevers (55, 56)], and which are a rich source of polyphenoloxidase, quantitative importance of the latter enzyme as a terminal oxidase seems contradicted by the very high affinity for oxygen and the considerable cyanide resistance of respiration (65).

No clear-cut evidence supports or denies the interesting possibility of a cytochrome-mediated oxidation of ascorbate. Honda (66), Arrigoni, Rossi & Marré (60), and Fritz & Naylor (67) reported *in vitro* oxidation of ascorbate by mitochondrial preparations, but the involvement of cytochrome oxidase could be demonstrated only in the presence of exogenous cytochrome-*c*, which non-enzymatically oxidizes ascorbate. Significant phosphorylation coupled to ascorbate oxidation—still in the presence of added cytochrome-*c*—was shown by Fritz & Naylor (67). As cytochrome-*c* seems strictly localized in mitochondria, to accept the hypothesis of a cytochrome-oxidase-mediated, phosphorylating ascorbate oxidation one should postulate the presence of an unknown intermediate electron carrier in plant cells, to substitute for cytochrome-*c* in connecting ascorbate with the endomitochondrial cytochrome chain.

A different approach to the problem of an involvement of the pentose phosphate pathway in phosphorylation comes from the finding [Servettaz & Marré (68), Servettaz (69), Forti *et al.* (70)] that some fraction of the total amount of the pentose phosphate pathway enzymes is bound to particulate elements of plant cell cytoplasm. Forti *et al.* (70) showed that the glucose-6-phosphate-oxidizing particles are different from true mitochondria in as much as they sediment at lower centrifugal forces, do not contain cytochrome oxidase and succinic dehydrogenase, and are endowed with considerable

TPN glutathione reductase, dehydroascorbic acid reductase, and ascorbic acid oxidase activity. The particle-bound ascorbate oxidase differs from that found in the supernatant in being strongly activated by ADP, and also, at a lower level, by 2,4-DNP. This suggested the involvement of a phosphorylating step; but neither net phosphate uptake nor ^{32}P-ATP exchange could be detected. It is true that the presence of a very active ATPase in this particulate fraction is a serious obstacle in these studies.

No direct evidence exists in favor of direct oxidation of extra-mitochondrial TPNH through the phosphorylating cytochrome chain, such as could be mediated by transhydrogenases, even though the presence of these enzymes in higher plants has been reported (71).

The Pool of Phosphate Transfer Potential

Since the first unequivocal demonstration by Albaum of the presence of adenosinepolyphosphates in higher plants, evidence has accumulated that these compounds represent the main Ptp reservoir in these organisms. No extensive research has been carried on concerning the existence of other auxiliary pools such as the creatine-phosphate and arginine-phosphate of animals. Inorganic polyphosphates and metaphosphates—which contain energy-rich phosphate bonds—are abundant in lower plants (72); but their presence in higher plants has been investigated to only a slight extent. The point is interesting, and further research in this field is desirable.

Besides ATP, other nucleoside polyphosphates playing an important role in phosphate metabolism (such as UTP and its sugar derivatives, and guanosine triphosphate) have been identified in higher plants (38). Among the enzymes catalyzing the reversible transport of phosphate between the various nucleotides, adenylic acid kinase has been partially purified and studied by various authors (73), and the presence of uridinemonophosphokinase and of uridinediphosphokinase in pea extracts has been reported (74, 75).

Utilization of Phosphate Transfer Potential

General.—Utilization of the Ptp stored in the ATP system follows a substantially common pattern in all organisms, and involves activation of substrates by kinases or phosphate transferases, catalysis of synthetic reactions by phosphorylases or pyrophosphorylases, and finally regeneration of inorganic phosphate by phosphatases.

Brilliant work with ^{18}O and ^{32}P (1) has demonstrated that in the kinasic and in the phosphatasic reactions a phosphoryl, rather than a phosphate group, is transported to the acceptor; while in phosphorylasic and pyrophosphorylasic reactions the P-O bond of phosphate remains intact. The former type of reaction may thus be considered as a nucleophilic attack on the terminal phosphorus atom of ATP by the acceptor, and the latter reaction as a nucleophilic displacement of the unphosphorylated moiety of the attacked molecule by phosphate or pyrophosphate. As far as phosphatases are concerned, several interesting investigations have followed the important

observation by Axelrod that some of these enzymes may efficiently transfer phosphate between organic compounds (76). Of particular interest in this regard seems the occurrence in higher plants of nucleoside transphosphorylases, catalyzing the reaction:

$$\text{nucleotide (a)} + \text{nucleoside (b)} \rightarrow \text{nucleoside (a)} + \text{nucleotide (b)}.$$

Recent evidence suggests that, at least under conditions of low water availability at the level of the reaction site, enzymes of the phosphatase group could be involved in transphosphorylations utilizing compounds endowed with a relatively low Ptp as phosphate donors (46).

Interconversion of carbohydrates and related compounds.—The scheme of carbohydrate interconversion presented in Figure 1 is based on the demonstration *in vitro* of the presence in higher plants of all of the enzymes involved, and is substantially supported by the evidence obtained through isotope studies *in vivo*. The scheme permits us to distinguish between three distinct, major pathways of sugar metabolism, communicating between each other at the hexose phosphate level and each characterized by the hexose phosphate from which it originates. The glucose-1-phosphate sequence involves the phosphorolytic mechanism of glucose-1-phosphate⇆starch interconversion, and the UTP⇆UDP sugar chain of reactions, leading to glucosides, xylose and xylosides, and pectins. The hexose-6-phosphate sequence involves the whole pentose phosphate pathway and is thought to lead to synthesis of nucleotidic pentose, and of various aromatic compounds including shikimic acid, the probable precursor of phenylalanine and tyrosine (77). The fructose diphosphate sequence involves the initial steps of glycolysis and of the mechanism of carbohydrate⇆organic acid interconversion.

From the point of view of distinguishing possible key points of control of metabolism by phosphate turnover, attention is called to the following points. The concentration of ATP and the activity of the various kinases must be of the utmost importance in determining the relative efficiency of the different pathways, a number of distinct enzymes in competition for ATP being involved in the scheme. The same consideration is valid for the UTP level and the relative activities of UMP and UDP kinases, and of the various UDP-sugar pyrophosphorylases. Pyrophosphorylasic syntheses are generally considered irreversible, as a consequence of hydrolitic cleavage of pyrophosphate by the widespread pyrophosphatases, as in the example:

$$(a)\ \text{UTP} + \text{glucose-1-phosphate} \leftrightarrows \text{UDPG} + \text{pyrophosphate}$$
$$(b)\ \text{pyrophosphate} + H_2O \rightarrow 2\ \text{inorganic phosphate}$$

where the reversible Reaction (*a*) is driven from left to right by the strongly exergonic Reaction (*b*) ($-\Delta F. = 7$kcal.). The evidence *in vivo* supports this view, since 1-^{14}C-labeled galactose and galacturonic acid fed to intact cells do not significantly contribute to respiratory CO_2, while they are rapidly incorporated into pectins and cell wall xylosides (78, 79, 80).

In pectin synthesis another important ATP-requiring step is probably involved, mediating the methylation of the galacturonic acid units. It is known that in yeast and in animals transmethylation requires the activation

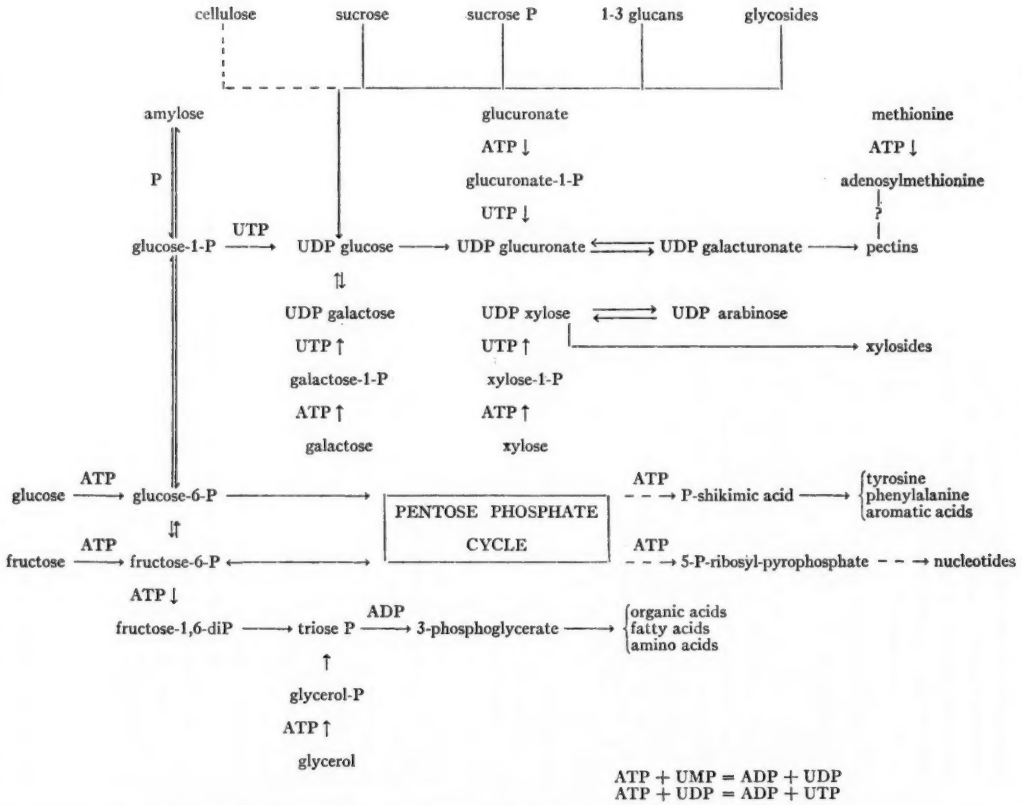

FIG. 1. Role of adenosine and uridine polyphosphates in carbohydrate interconversion in higher plants.

by ATP of the methyl donor methionine:

ATP + methionine → P_i + pyrophosphate + S—adenosylmethionine (81, 82),

adenosylmethionine being the general methylating agent. In higher plants methyl groups of methionine have been shown to be employed for the methylation of the uronic units of pectins in a transmethylation reaction [Sato *et al.* (83)]. The recent finding by Mudd, that barley extracts catalyze both the synthesis of S—adenosylmethionine (84) and the methyl transfer from this compound to suitable acceptors (85), suggests that such a mechanism could also be involved in pectin synthesis.

Glycolysis.—The chain of reactions leading from triose phosphate to pyruvate includes three reactions in which phosphorylation or transphosphorylation are involved. The coupling of glyceraldehyde-phosphate oxidation and DPN reduction to ATP synthesis from ADP makes this reaction easily reversible. The relation of this fact to the control of metabolism by phosphate-turnover will be discussed in a further section.

Phosphorylation of ADP by phospho*enol*pyruvate and pyruvate kinase is a largely exergonic reaction; however, recent studies by Krimsky (86) on a cell-free reconstructed system suggest that it could be reversed, even *in vivo*, under conditions of high ATP/ADP and pyruvate/phospho*enol*pyruvate ratios. For the use of pyruvate in the reversal of glycolysis, Krebs [see (87)] has postulated an ingenious ATP- and TPNH-involving mechanism:

(*a*) pyruvate + CO_2 + TPNH → malate (malic enzyme)
(*b*) malate + DPN^+ → oxalacetate + DPNH
(*c*) oxalacetate + ATP → CO_2 + phosphoenol pyruvate + ADP.

All the enzymes of this system have been shown in higher plants and recent studies consistently support the idea that Reaction (*c*) is the main route of reversal of glycolysis in sugar synthesis from fatty acids during fatty seed germination (88, 89). Finally, phosphorylation may play an indirect role in the interconversion between the triose phosphates as Stumpf (14) demonstrated that higher plant extracts may catalyze the sequence:

(*a*) dioxyacetone-phosphate + DPNH → glycerolphosphate + DPN^+
(*b*) glycerolphosphate + 1/2 O_2 (mitochondria) → glyceraldehyde-3-phosphate + H_2O

as mitochondrial oxidation of glycerolphosphate is coupled to phosphorylation.

Amino acid metabolism.—Recent work on animal materials and microorganisms shows that peptide bond synthesis involves as a first step the activation of amino acids by an ATP-dependent pyrophosphorylasic reaction: (*a*) ATP+amino acid→amino acyl—adenylate+pyrophosphate, followed by RNA requiring polymerization of the activated amino acyl units (7, 16). Studies of this mechanism are largely based on the demonstration *in vitro* of an exchange between radioactive pyrophosphate and ATP, such as should be expected when Reaction (*a*) is occurring. Amino acid-dependent ATP-pyrophosphate exchange accompanied by amino acid incorporation into proteins has been demonstrated in cell-free preparations from a number of plant tissues (90, 91, 92).

ATP is also required for glutamine synthesis from ammonia and glutamic acid [Webster (93)] and for activation of shikimic acid by shikimic kinase during the biosynthesis of phenylalanine and tyrosine [Davies (77)].

Fat metabolism.—Several reports show that the synthesis of fatty acids from acetate and the activation of fatty acids is also an ATP and CoA requiring process in higher plants. Stumpf recently reported palmitate and oleate synthesis by a clear extract from avocado particles, in the presence of ATP, CoA, and TPNH. The mechanism of the acetate activating reaction seems to be, as in animals and microorganisms, pyrophosphorylasic (94). The presence in peanut mitochondria of an enzyme catalyzing phosphorylation of α,β-diglycerides and α-monoglycerides has been reported by Bradbeer & Stumpf (95).

ATP is necessary in lipid breakdown for the initial fatty acid activation, and also for glycerol phosphorylation. The enzyme catalyzing this reaction, glycerol kinase, has been recently demonstrated by Stumpf in pea cotyledons. An ATP requirement for phospholipid synthesis in mitochondrial preparations has been recently reported [Stumpf (96)].

Researches by Shneour & Zabin on lycopene synthesis in cell-free preparations from tomato fruits indicate that mevalonic acid and ATP are involved in carotenoid synthesis (97).

Sulfur metabolism.—It has been shown (98 to 100) that activation of sulfate in yeast involves two steps:

(*a*) ATP + sulfate ⇆ adenosinephosphosulfate (APS) + pyrophosphate
(*b*) APS + ATP → 3-phosphoadenosine-5-phosphosulfate + ADP

Reaction (*a*) is endergonic, but is driven from left to right by both pyrophosphate hydrolysis and by the large free energy change of Reaction (*b*). The enzyme catalyzing Reaction (*a*) has been named ATP sulforylase. Selenate can substitute for sulfate, adenosine phosphoselenate being formed (100). In this connection the recent finding of selenate-containing amino acids in *Astragalus* seed proteins is of interest (101). The enzyme ATP sulphorylase has been found in higher plants (Squire & Bandurski, personal communication). Reduction of active sulfate 3-phosphoadenosine-5-phosphosulfate to sulfite has been demonstrated in yeast extracts (102). According to recent studies, TPNH (103) or lipoic acid (104) may function as electron donors in this reaction.

Phosphate Transfer Potential Utilization in Physiological Processes

While the dependence of the above discussed mechanisms on Ptp energy has been demonstrated at the molecular level, the involvement of phosphorylation in a number of physiological processes is strongly suggested by observations *in vivo*.

Active uptake of solutes, growth by cell extension, and control of the rate of respiration, for which more data are available, will be briefly discussed here.

Solute uptake.—The relationship between respiration and ion absorption, and the problem of whether active ion uptake depends directly on electron transfer, or on Ptp generated by oxidative reactions, has been recently discussed by Robertson (105), Laties (106), and Sutcliffe (107). A few additional remarks on the possible role of phosphorylation in this process may be made.

The main indication in favor of the requirement of Ptp for ion uptake is its inhibition by uncoupling agents such as 2,4-DNP. Robertson (105) suggested that the DNP effect could depend on the fact that electron transfer is effective for active ion uptake only when it proceeds coupled to phosphorylation, even if ATP synthesis is not the determining factor. This interpretation, however, is difficult to test experimentally and remains purely hypothetical.

Recent evidence indicates that ion uptake is inhibited not only by uncoupling agents that do not change the pathway of electron transfer, but also by cytochrome oxidase inhibitors, such as cyanide, CO, etc., even in the cases in which overall oxygen uptake is not affected. Hackett and co-workers (108) report inhibition of inorganic phosphate uptake by KCN in aged potato disks, respiration of which is largely KCN insensitive, while Shealtiel and Ducet (109) find that CO inhibits Rb absorption by barley roots by 75 per cent, without affecting oxygen uptake. Active amino acid uptake by carrot slices has been shown to be strongly inhibited by DNP as well as by KCN [Burt & Hird (110, 111)].

On the other hand, not only active ion uptake, but also the uptake of non-ionized compounds, such as sugars, is inhibited both by pure uncouplers and by inhibitors of cytochrome oxidase. Pennell & Weatherley found that KCN, azide, and DNP, as well as anaerobiosis, inhibit the penetration of sugars into leaf disks; sugar uptake was the same in air and in 2 per cent oxygen although O_2 uptake was inhibited by 35 per cent (112). Similarly, the rapid uptake of labeled glucose by aged potato disks is strongly inhibited by catechol at concentrations inhibiting respiration by 25 per cent, while a decrease of oxygen to 4 per cent, though decreasing respiration to the same or larger extent, does not affect sugar uptake at all (113).

In animal cells and in microorganisms, sugar uptake against a concentration gradient has been shown to be completely inhibited by uncoupling agents [Crane & Crane (114)].

The above evidence, together with the difficulty of establishing a strict quantitative correlation between solute uptake and solute-dependent oxygen uptake (106), strongly suggests that some common basic Ptp energy-utilizing mechanism is required for the active uptake of any kind of solute. This of course does not contradict the evidence in favor of the existence of specific carriers or mechanisms for the uptake of the different molecular species, as the very synthesis or functioning of these carriers or mechanisms could depend on Ptp. In this connection, Laties' results on chloride uptake by potato slices at different temperatures may be quoted (115); Laties found that in aging disks the capacity for chloride uptake rapidly increases, the increase being reversibly inhibited either by low temperature or by 2,4-DNP. As both conditions obviously induce a lowering of Ptp in the cells, these data

would be easily interpreted by the assumption of a Ptp energy-dependent synthesis or activation of a carrier. It may be pertinent to mention here that carrier synthesis has been demonstrated in bacteria as a substrate-inducible phenomenon in the case of galactose permease (116).

Growth by cell extension.—Growth by cell division and protoplasm synthesis is an obviously Ptp-dependent process in several essential aspects, such as protein and nucleic acid synthesis. The requirement for Ptp energy in growth by cell extention is less clearly understood. A number of facts indicate that treatments inhibiting phosphorylation also inhibit cell extension (117). Carbon monoxide and cyanide inhibit water uptake even when, as in aged potato disks, they do not affect oxygen uptake (118). The simplest interpretation of these results is that the level of Ptp conditions this type of growth. Analysis of the mechanism of cell extension suggests that the metabolism of some component of the cell wall is essentially involved, as cell wall plasticity increases during cell extension. Recent important work shows that the metabolism of a pectin fraction is strongly enhanced during hormone-induced stimulation of cell extension (119–121). The dependence of cell extension on methylation of pectin is also indicated by its rapid inhibition by ethionine—which blocks methyl transfer from methionine to pectin—and by the complete reversal of the inhibition by methionine [Cleland (122)]. If one accepts the idea of a central role of pectin anabolism in cell extension, the necessary dependence of this type of growth on Ptp appears as a logical corollary. As discussed in a preceding section, ATP is required in two, or quite probably three, steps of pectin biosynthesis: hexose (or uronic acid) phosphorylation, UTP synthesis for UDP-sugar formation, and activation of methionine. Similarly, the synthesis of other cell wall components, as xylosides [and possibly cellulose (123)] utilizing the UDP-sugar pathway, is obviously Ptp energy-dependent. It is thus understandable that no growth by cell extension can be observed when the Ptp level in the cells falls below a threshold value. This seems to provide the more likely explanation for the inhibitory effect on growth by all respiratory inhibitors and uncouplers. On the other hand, it seems clear that such a basic involvement of Ptp energy in growth does not imply that hormonal regulation of the same process is necessarily mediated by an effect on phosphorylative systems [cf. Marré *et al.* (29, 124)].

The Control of Respiratory Metabolism by the Phosphorylating System

As essential phosphorylative steps are involved in practically all metabolic pathways, it appears obvious that the efficiency of enzymes and the level of substrates involved in phosphorylation reactions closely control almost any important process in the cell. To restrict the field to a single, basic point, only the relationship between glycolytic and respiratory activity and ATP, ADP, and inorganic phosphate levels will be dealt with here. The problem has already been discussed by Laties (125), Axelrod & Beevers (10), and Hackett (11).

The suggestion by Lardy (126) that the level of ADP and inorganic phosphate could control the rate of oxidative reactions *in vivo* has found support in a number of experiments using widely varied materials. The possibility that this mechanism may operate is based on the assumption that in the whole cell—as is usual in isolated mitochondria—oxidations must be tightly coupled to phosphorylations; thus the rate of O_2 uptake should depend on the availability of ADP and inorganic phosphate which, on the other hand, depends on the rate of transphosphorylations utilizing ATP as phosphate donor.

The physiological importance of such a mechanism would be that of equating at any particular moment the activity of the oxidative machinery to the requirement for Ptp (that is, for ATP high energy bonds) for biological work.

Highly interesting experimental analyses of the problem have recently been made by Gatt & Racker (127, 128), by Wu & Racker (40, 129), and by Wu (37). Gatt and Racker, using a reconstructed system containing mitochondrial and glycolytic enzymes, found that when both systems are operating a strong competition for both ADP and inorganic phosphate between glycolysis and mitochondrial oxidations appears. In the inhibition of respiration by glycolysis, the more important factor seems to be ADP, while inorganic phosphate is rate limiting only at very low concentrations (less than $3\times10^{-4}M$). In contrast, glycolysis requires, for full operation, much higher concentrations of inorganic phosphate, as for concentrations lower than $5\times10^{-3}M$ a strong inhibition of both glucose phosphorylation and lactate synthesis by mitochondrial oxidations is observed. This suggests that even *in vivo* determinations of O_2 uptake could make evident only a state of competition for phosphate acceptors, while the detection of competition for inorganic phosphate would require the determination of glycolytic activity.

The main conclusions of this work *in vitro* have been tested *in vivo* by Wu and Racker, on isolated ascites tumor cells. In this material, glycolysis is increased either by anaerobiosis or by 2,4-DNP by about 80 per cent, either treatment inducing a marked rise of inorganic phosphate, while no significant changes of the ATP/ADP ratio are observed. The addition of inorganic phosphate to the medium under aerobic conditions increases both glycolysis and glucose uptake, while the addition of AMP was ineffective on these processes, although it increased the level of internal ATP, ADP, and AMP. The observation that the apparent internal concentration of inorganic phosphate was about $9\times10^{-3}M$, while the affinity constant of 3-phosphoglyceraldehyde dehydrogenase is of about $1.5\times10^{-3}M$, leads the authors to suggest that some cell compartmentalization factor makes inorganic phosphate incompletely available for this enzyme, and that this condition could be attenuated to some extent during anaerobiosis.

An important role of compartmentalization of phosphate intermediates in the cell is also suggested by the work by Chance & Hess (41) who determined spectrophotometrically the state of oxidation of the electron carrier

chain in short time experiments in intact, freshly prepared ascites tumor cells. Additions of glucose to the medium induced at first a rapid rise of respiration and the oxidation of mitochondrial carriers, immediately followed by a period of inhibition of O_2 uptake, concomitant with the return of cytochrome-*b* to a state of reduction equal to or slightly higher than the initial. The suggested interpretation is that in the initial phase ADP increases because of hexose phosphorylation, while in the following period it decreases because of the slow rate of diffusion of the ATP synthesized at the mitochondrial level (mitochondrial ATP store) to the site where it would be used for hexose phosphorylation, and thus regenerate ADP. These results clearly emphasize the dominant role of ADP in the control of oxidative metabolism. They do not seem, however, to contradict the evidence brought by Racker and coworkers in favor of the importance of inorganic phosphate level for the control of glycolysis.

A number of data indicate that also in higher plants ATP, ADP, and inorganic phosphate levels may control glycolytic and oxidative metabolism. Many reports [see Hackett (11) and also Gaur & Beevers (130)] show that plant mitochondria require ADP and inorganic phosphate, or uncoupling by 2,4-DNP or other substituted phenols to show full oxidative efficiency. Hatch & Turner (131) demonstrated that the operation of typical glycolysis in an acetone powder extract from pea seeds requires inorganic phosphate and adenine nucleotides.

In vivo, the knowledge of the role of phosphate-acceptor level in the control of respiration is based on a large number of experiments with uncouplers, and also on data on glycolysis activation by anaerobiosis (Pasteur effect). The important work by Beevers (132) showing that DNP increases, besides O_2 uptake, the R.Q. and the alcohol and acetaldehyde production has been confirmed and extended (10, 11, 13). Beevers' suggestion, that the DNP-induced increase of fermentation is an indirect effect of the uncoupling of phosphorylation at the mitochondrial level, is further supported by studies showing that in pea internode segments DNP induces, together with the increase of alcohol and acetaldehyde production, a strong decrease of high energy phosphate and of hexose phosphate [Bianchetti & Marré (133)]. It is interesting that the observed decrease of Norit A charcoal-adsorbable nucleotide (high energy phosphate) is significantly larger than what could be accounted for by the amount of ATP (enzymatically determined) present in the controls. This seems to indicate not only that all of the ATP initially present was converted to ADP, but also that some ADP was converted to AMP, presumably because of the activity of adenylic kinase. If this were true, one could imagine that the inhibition of glycolysis observed at very high (superoptimal) DNP concentration could at least in part depend on the decrease of ADP occurring when ATP concentration approaches zero. On the other hand, this hypothesis does not explain the inhibitory effect of high DNP concentrations on O_2 uptake, as oxidative metabolism of isolated mitochondria is strongly inhibited by DNP concentrations higher than $10^{-4}M$ even in the presence of a phosphate acceptor system (130). It seems

reasonable to ascribe this effect to the depletion of the endo-mitochondrial store of ATP, as ATP is an efficient protective agent for mitochondrial structures (134, 135). Eliasson has recently suggested that the ATP deficiency induced by uncouplers could explain the progressive inhibition of O_2 uptake in intact tissues treated with DNP (136, 137). It could be observed, however, that in whole cells many factors are simultaneously affected by uncoupling at the mitochondrial level; among others, the marked decrease of the rate of phosphorylation of hexoses could play a determining role (133).

An important corollary of accepting the view that the DNP effect on fermentation is an indirect consequence of the uncoupling of mitochondrial phosphorylations, then, is that it would be possible to obtain strong activation of glycolysis—or of other ADP and inorganic phosphate dependent respiratory mechanisms—even under physiological conditions during which oxygen uptake is unaffected. It has recently been found by Sugiura in Oota's laboratory (personal communication) that while pea cotyledons respond to DNP in the usual way—that is by activation of both CO_2 production and O_2 uptake—in the pea germ axis the uncoupler stimulates only CO_2 production while O_2 uptake steadily decreases for increasing concentrations of DNP. The interpretation proposed is that in the germ axis the efficiency of ATP-utilizing mechanisms is high enough to allow the maximal rate of mitochondrial oxidations, while the strong DNP-induced increase of ADP—and one would add, in view of the above discussed data by Wu & Racker, perhaps also of inorganic phosphate—may still affect glycolytic CO_2 production.

An interesting analysis of *in vivo* changes of adenine nucleotides and phosphate esters in relation to the Pasteur effect in pea seeds has been recently reported by Rowan *et al.* (35). These authors found that anaerobiosis induces in this material, together with the usual increase of CO_2 output, a definite increase of inorganic phosphate and ADP, and a decrease of hexose-phosphate. This picture is similar to that observed in the above quoted work by Wu & Racker during anaerobiosis in the ascites tumor cells, where the effect on glycolysis is interpreted as a consequence not only of ADP increase, but also of inorganic phosphate increase. The point seems worth emphasis, as the importance of phosphate level changes for metabolic control is usually underestimated, relative to the importance ascribed to phosphate acceptor availability.

An interesting alternative mechanism for the interpretation of the Pasteur effect has been recently suggested by Hatch & Turner, who found that in a dialyzed extract from pea seed acetone powder glycolysis was strongly inhibited by the presence of oxygen (138). Inhibition by oxygen was accompanied by a rapid decrease of protein sulfhydryl groups, and it was completely reversed by high concentrations of either DPN or glutathione. This leads these authors to suggest that even *in vivo* the oxidation of the essential sulfhydryl of triose phosphate dehydrogenase, in air, and the reductive reactivation of the enzyme under anaerobic conditions might be put in relation with the Pasteur effect. These data are certainly interesting from several aspects, including that of the control of this key step in carbohydrate break-

down; however, the participation of such a mechanism in the Pasteur effect seems doubtful. The oxidative inactivation of triosephosphate dehydrogenase observed in these experiments was largely prevented by Versene, and appears a non-enzymatic, metal ion catalyzed process, qualitatively similar to the often occurring and most annoying inconveniences one meets in enzyme work; thus the occurrence of the phenomenon *in vivo* seems highly hypothetic. Moreover, the strong activation of glycolysis by DNP, usually found *in vivo* under aerobic conditions, seems to indicate that the activity of triosephosphate dehydrogenase per se is not a severely limiting factor for this process; in fact, there is no reason to suppose that DNP could increase in the cell the amount of DPNH or other reducing agents capable of bringing the triosephosphate dehydrogenase to its fully active state.

Physiological uncoupling.—The important idea of the possibility of a physiological uncoupling of mitochondrial oxidations from phosphorylations has been postulated by Hock and Lipmann, on the basis of the finding that thyroxin-treated mitochondria are completely insensitive, as far as their oxidative capacity is concerned, to the absence of phosphate acceptors while in the presence of ADP and inorganic phosphate they show phosphorylative and oxidative capacity identical to that of normal, tightly coupled mitochondria (139). Thyroxin thus induces, under these experimental conditions, a facultative uncoupling of phosphorylation. A similar possibility of physiological, facultative uncoupling has been recently postulated by Gatt & Racker (128). They suggested that the inhibition of mitochondrial ATPase by ADP and its requirement for a very high ATP level for maximal activity could result, even *in vivo*, in a situation in which, when the level of ATP rises and that of ADP falls to a very low value, the activation of mitochondrial ATPase would partially uncouple phosphorylation thus allowing the production of metabolically important substrates by oxidative reactions.

Several data indicate the presence in higher plants of physiological uncoupling mechanisms of this type. Forti showed that plant mitochondrial ATPase is also strongly inhibited by ADP, and requires a high ATP concentration for significant activity (140). Other, presumably more important mechanisms of facultative uncoupling might be deduced by the important studies on cyanide and carbon monoxide resistant respiration, reported by Hackett (11, 108), Chance & Hackett (141), Ducet (142), and by a number of other authors. It has been shown in many cases—including those of aged slices from storage tissues and of leaf disks—that even when respiration is not inhibited, the inhibitors still block almost any process that, directly or indirectly, depends on a high level of phosphorylative efficiency. This is the case with cell extension, phosphate uptake and incorporation into organic compounds, protein synthesis, and sugar uptake in the aged potato disks, and of salt and sugar uptake by leaf disks. Quite interestingly, a common feature of the materials showing this effect is insensitivity to CO, even when cytochrome oxidase is present in the isolated mitochondria (143, 144), and also insensitivity to uncoupling agents such as DNP (55, 56, 108). The discovery that in two of the tissues giving this response, the skunk cabbage spadix and the

aged potato disks, mitochondria contain an apparently autoxidizable cytochrome, cytochrome-b_7, besides the normal cytochrome chain, and the finding that in both cases cyanide does not inhibit O_2 uptake, though it blocks phosphorylation (111, 144, 145) led Hackett to suggest that two different electron pathways are operating for the oxidation of mitochondrial substrates. The first of these, cytochrome-a_3-mediated and phosphorylating, would operate in the absence of inhibitors; the second, utilizing cytochrome-b_7 as terminal oxidase, would become active only when the cytochrome oxidase pathway is blocked (11).

The importance of these data and the hypothesis in regard to the problem of facultative uncoupling of phosphorylation is obvious. It seems probable that under conditions in which a high ATP/ADP level would inhibit oxidative metabolism in a tightly coupled system, the existence of an uncoupled, auxiliary pathway would provide a possibility for oxidations to proceed. This pathway would become active as soon as an ADP shortage would close the a_3 system. Also, the fundamental difference between this type of uncoupling and that induced by DNP is evident; in the former case ATP is regularly synthesized in the amount required by Ptp dependent reactions, while in the latter case mitochondrial ATP synthesis is suppressed. It is interesting to observe that the reported increases of CO_2 output—and perhaps also of O_2 uptake—in tissue treated with cytochrome-a_3 inhibitors could also be explained through this mechanism. The rise of the level of ADP and inorganic phosphate would presumably stimulate glycolysis and also supply a larger amount of respiratory substrates to the carboxylic cycle.

The more obvious physiological interest of such a mechanism seems to consist in the release of synthesis of anabolites from the restraint imposed on oxidative metabolism by phosphate-acceptor availability. It seems interesting that among the materials showing facultative uncoupling one finds aged slices from storage tissue and green leaves. In the former material, protein synthesis is usually observed (146). This requires a corresponding rate of synthesis of amino acids and some ATP; but formation of amino acids such as glutamic and aspartic acids from hexoses would imply the synthesis of more high energy phosphate bonds than would be utilizable for the synthesis of the corresponding peptide bonds. Thus, in the absence of a facultative uncoupling mechanism, protein synthesis could be—at least under some conditions—significantly slowed down by the dependence of oxidative breakdown on ADP availability.

The case of mature green leaves is perhaps even more interesting. Oxidative breakdown in these tissues is required for the elaboration of photosynthetic products, and active protein synthesis in the light is often observed. Now it has been reported [Kandler (147)] that—especially under conditions of low CO_2 availability—the ATP/ADP+inorganic phosphate value in illuminated green tissues reaches very high values because of photosynthetic phosphorylation. Even if one assumes that some cell compartmentalization limits the rate of intracellular redistribution of adeninenucleotides, it appears quite probable that under these conditions the amount of

ADP available for glycolysis and mitochondrial oxidations is drastically decreased. The physiological, facultative uncoupling of phosphorylations in these tissues it seems could thus be a requirement to allow oxidative carbohydrate breakdown to proceed even when photosynthetic phosphorylation tends to reduce the level of phosphate acceptor to a very low value. In this connection also, the otherwise rather puzzling report by Arnon of the specific localization in green leaves of an inorganic phosphate and ADP independent triosephosphate dehydrogenase could find a physiological interpretation.

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BIOSYNTHESIS AND FUNCTION OF CAROTENOIDS[1,2]

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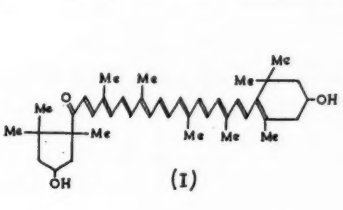
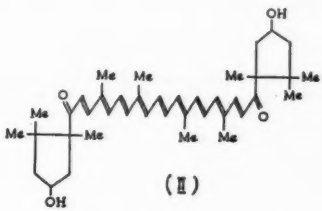
Carotenoids are widely distributed plant pigments and the problems of their formation and function have interested biochemists and plant physiologists for a considerable time; so it is with these two aspects that this review is concerned. But before considering these it is appropriate to point out that during the last two or three years fascinating developments have been made in carotenoid chemistry. For example, Isler and his colleagues (1) have devised elegant total syntheses of many carotenoids; Weedon and his colleagues (2) have proved that capsanthin and capsorubin, the characteristic carotenoid pigments of the red pepper *Capsicum annuum*, have pentanoid rings (I,II); and Yamaguchi (3, 4) has shown that the two pigments renieratene (III) and isorenieratene (IV), obtained from the sponge *Renieria japonica*, contain aromatic ring systems. The structure of isorenieratene has been confirmed by total synthesis (5, 6).



(I)



(II)

(III)

(IV)

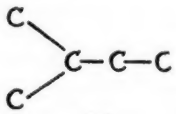
BIOSYNTHESIS OF CAROTENOIDS

The carotenoids represent one class of a very wide group of natural products known collectively as terpenoids. Their common characteristic is that they are built up from isopentane (isoprene, branched 5-C) units (V). In

[1] The survey of literature pertaining to this review was completed July 31, 1960.

[2] The following abbreviations will be used: AMP (adenosine-5′-phosphate); DMA (dimethylacrylic acid); DMA-CoA (β,β-dimethylacrylyl-coenzyme A); DPA (diphenylamine); DPN (diphosphopyridine nucleotide); HMG (β-hydroxy-β-methylglutaric acid); HMG-CoA (β-hydroxy-β-methylglutaryl-coenzyme A); IPP (isopentenylpyrophosphate); MVA (mevalonic acid).

mono- (two 5-C units), sesqui- (three 5-C units) and di- (four 5-C units)



(v)

terpenoids the 5-C units are joined head to tail throughout the molecules; on the other hand, the tri-terpenoids (six 5-C units) which include the steroids, and the tetraterpenoids (eight 5-C units), the carotenoids, appear to arise by a tail to tail condensation of two identical units of 15 carbon atoms and 20 carbon atoms respectively. In investigating the biosynthesis of the terpenoids one has to consider (*a*) the nature and formation of the 5-C polymerizing unit, (*b*) the nature of the polymerization of the 5-C units, (*c*) the mechanism of the "tail to tail" condensation in the tri- and tetraterpenoids, and (*d*) special problems connected with each class. In the case of carotenoids these include (*i*) cyclization, which is not as extensive as in the triterpenoids, (*ii*) a high degree of unsaturation, which is very characteristic of carotenoids, (*iii*) *cis-trans* isomerization, and (*iv*) the introduction of various oxygen functions. These various aspects will be considered in turn.

Nature and Formation of the 5-C Polymerizing Unit

The degradation of β-carotene, synthesized by the mould *Mucor hiemalis* in the presence of either [1-^{14}C]- or [2-^{14}C]-acetate as the sole carbon source, revealed an isotope distribution (Fig. 1) (7, 8, 9) which is typical of all other terpenoid derivatives (10). [The report that the isotope distribution was different in lycopene produced by ripening tomatoes (11) has not been con-

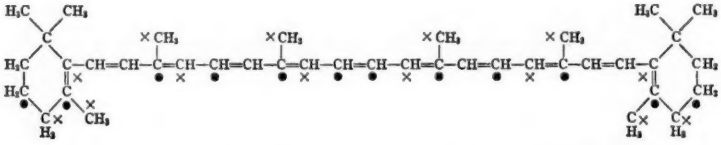
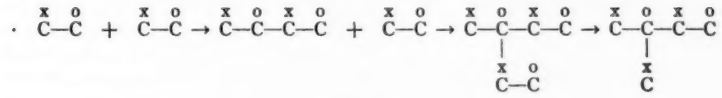


FIG. 1. Distribution of labelled acetate in β-carotene from *Mucor hiemalis*. ● indicates carbon atoms arising from the methyl carbon of acetate and x the carbon atoms arising from the carboxyl carbon of acetate.

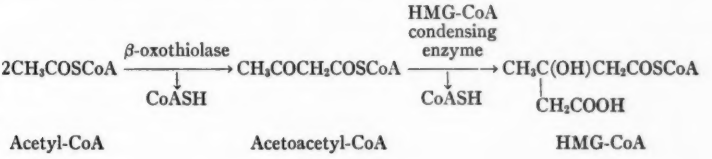
firmed (12).] Results identical with those reported for *M. hiemalis* were obtained using the closely related mould *Phycomyces blakesleeanus* and adding the labelled acetate to cultures growing on a glucose/asparagine medium (13, 14). The lack of significant randomization of [2-^{14}C]-acetate in the carotene molecule indicates that the tricarboxylic acid cycle plays no direct part in carotenoid biosynthesis; this is also true for other terpenoids (10, 15). The

observed labelling pattern could theoretically be achieved by condensation of three acetate molecules, as indicated in Scheme 1, to yield a 6-C acid as the



SCHEME 1.

first branched compound in the terpenoid pathway; this acid would then yield a 5-C compound by decarboxylation. Rudney and Lynen and their collaborators (16, 17, 18), using purified liver and yeast enzyme systems, have shown that the first 6-C compound formed is β-hydroxy-β-methylglutaryl-CoA (HMG-CoA) (Scheme 2).



SCHEME 2.

Confusion has arisen over HMG as a precursor of terpenoids; [^{14}C]-HMG-CoA can be incorporated into terpenoids (16) whereas free [^{14}C]-HMG is insignificantly incorporated into steroids (19, 20). This is due to the fact that an HMG-activating enzyme converting HMG to HMG-CoA is not present in yeast or liver. It appears that the activity of HMG in diluting out the incorporation of [^{14}C]-acetate into cholesterol (21) is not a true isotope dilution effect but is due to HMG acting as an antimetabolite, for Wright (22) showed that HMG strongly inhibited the growth stimulation of *Lactobacillus acidophilus* by mevalonic acid (MVA). However, the incorporation of [2-^{14}C]- and [3-^{14}C]-HMG into β-carotene and ergosterol has been reported using both intact cells and cell-free systems of *P. blakesleeanus* (23, 24).

The idea was sponsored for some time that HMG-CoA was converted by decarboxylation and dehydration into β,β-dimethylacrylyl CoA (DMA-CoA) (Scheme 3), which was the true 5-C polymerizing unit for carotenoid

SCHEME 3.

biosynthesis; and indeed [3-^{14}C]-DMA-CoA is incorporated into β-carotene in the alga *Chlorella pyrenoidosa* and the mould *Blakeslea trispora*, but not into lycopene in ripening tomatoes (25). However, the recent investi-

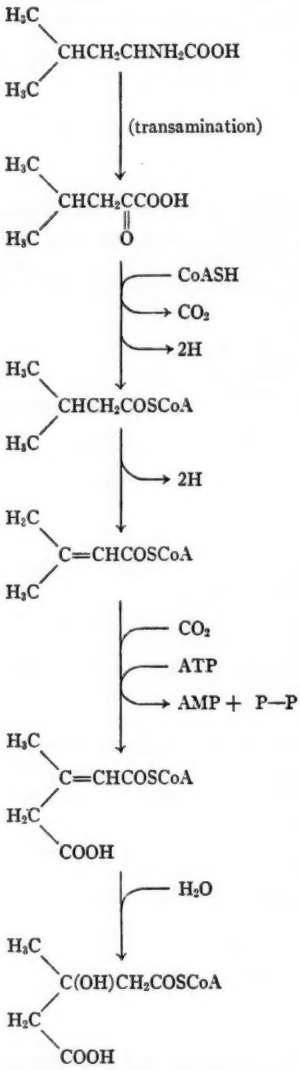
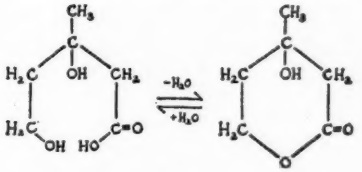


FIG. 2. Formation of HMG-CoA from leucine.

gations of Coon and his colleagues on the metabolism of leucine in animal tissues (26) and of Lynen and his group on carboxylation reactions in microorganisms and animal tissues (27, 28) indicate that DMA-CoA can be converted into HMG-CoA but that the reverse reaction is not possible (Fig. 2). Furthermore, the labelling pattern observed in β-carotene formed by *P. blakesleeanus* in the presence of leucine labelled with ^{14}C in various carbon atoms (see p. 225) is not compatible with the metabolism of leucine to DMA-CoA and the direct incorporation of this substrate into β-carotene.

A discovery that changed the situation almost overnight was that mevalonic acid (VI), the acetate replacing factor in *L. acidolphilus* ATCC 4963 (29), was a most potent precursor of cholesterol in rat liver homogenates and superior to all other compounds tested (19, 30). These observations were soon extended to other terpenoids including carotenoids; in particular MVA was found to be actively incorporated into β-carotene by *P. blakesleeanus* (14, 24, 31, 32), *M. hiemalis* (33), *B. trispora* (25), and carrot root preparations (34), and into lycopene in ripening tomatoes (13, 14, 35). It is not incorporated into β-carotene by *C. pyrenoidosa* (25) and incorporated only to a slight extent into spirilloxanthin by both intact cells and isolated chromatophores of *Rhodospirillum rubrum* (36, 37). All these results are almost certainly due to a permeability barrier. Purcell *et al.* (38) misinterpreted a statement of the reviewer in commenting that "Braithwaite & Goodwin have concluded that mevalonic acid is not effectively incorporated into β-carotene by *Phycomyces blakesleeanus*."

Under comparable conditions [^{14}C]-mevalonate is some 15 to 20 times more



(VI)

effectively incorporated into carotenoids than is acetate (12, 33, 35). HMG-CoA, formed as indicated in Scheme 2, is converted into mevalonic acid and the reaction, which has been studied mainly in yeast, can take place in stages via mevaldic acid [(A)&(B), Fig. 3] or in one step [(C), Fig. 3] without the formation of free mevaldic acid (39, 40, 41). The two-step process is catalysed by mevaldic acid dehydrogenase (A), a particulate enzyme requiring TPN as hydrogen acceptor and mevalonic acid dehydrogenase, which is also TPN-dependent. By the use of [1-^{14}C]- and [2-^{14}C]-mevalonate it has been possible to demonstrate that it is the carboxyl group esterified with CoA which is reduced to the terminal alcohol group of mevalonic acid (Fig. 3) (39). Experiments on cholesterol biosynthesis with [1-^{14}C]-mevalonate soon demonstrated that C-1 was lost during the incorporation of mevalonate into the terpenoid residue (30) and experiments with [2-^{14}C]-mevalonate showed that in both sterols (30, 42) and carotenoids (13, 14) C-2 and C-3′ retained

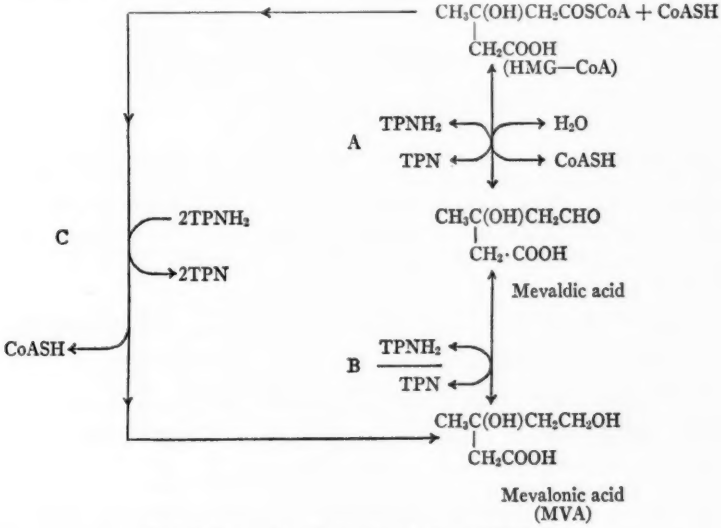
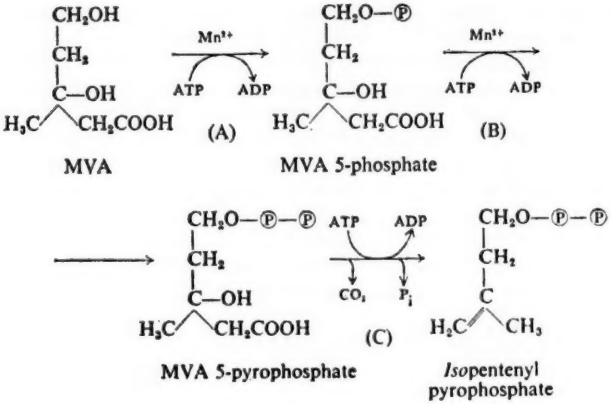


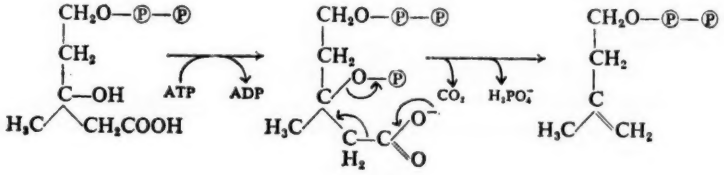
FIG. 3. Formation of mevalonate from HMG-CoA.

their individuality. That is, when C-1 is lost, the resulting *gem* dimethyl groups of the repeating 5-C unit are not biologically equivalent. How this is achieved has been revealed by Bloch & Lynen and their colleagues who have been concerned mainly with sterol biosynthesis. Their work has revealed that mevalonate is converted into isopentenylpyrophosphate (IPP), which now appears to be the fundamental repeating unit for the biosynthesis of terpenoids (see 43). It has been converted into sterols and rubber by appropriate enzyme preparations (44), but an experiment with carotenoids has not yet been reported; however, there is no reason to doubt that the same result would be obtained. IPP is formed by the reactions indicated in Scheme 4.



SCHEME 4.

Reaction A is catalysed by mevalonic kinase, which has been purified some 200-fold from yeast (45); enzymes carrying out reactions (B) and (C) have also been purified from yeast and liver (46, 47, 48). It should be emphasized that in reaction (C), CO_2 and inorganic phosphate (P_i) are eliminated together, and a suggested concerted mechanism for the reaction is given in Scheme 5 (47).



SCHEME 5

Leucine and carotenogenesis.—It was discovered some years ago that leucine stimulated carotenogenesis in *P. blakesleeanus* (49). However, the failure of

[2-^{14}C]-leucine to be incorporated into β-carotene (50) indicated that the conventional pathway via DMA (Fig. 2) was not functioning to any considerable extent. During the past few years Mackinney and his colleagues (23, 50, 51, 52, 53) have further investigated this problem in detail, especially with the aid of leucine labelled in different carbon atoms. The relative specific activities in β-carotene derived from various [^{14}C]-leucines were C-5 (7.6), C-4 (8.7), C-3 (3.6), C-2 (0.26), and C-1 (0.05). These results can be explained by the metabolic scheme given in Figure 4. This allows for (*a*) the incorporation of C-5, 5′, and C-4 as a unit, (*b*) the relatively weaker incor-

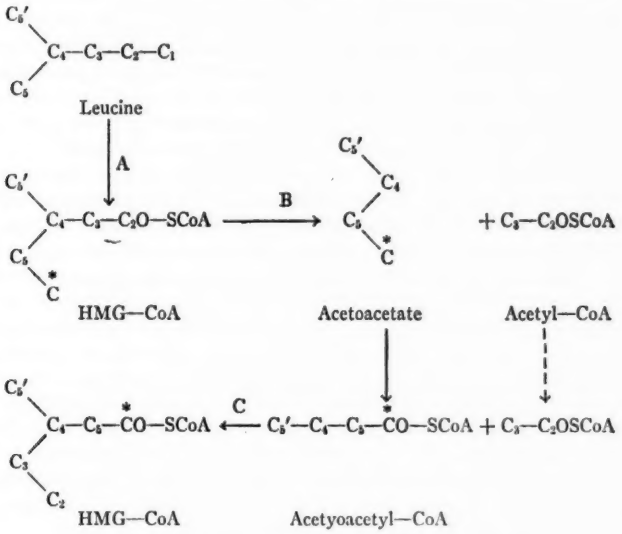


FIG. 4. The conversion of leucine into carotenoids
(The starred carbon atom arises from CO_2).

poration of C-3 compared with C-5, 5′, and C-4 [following reaction (B), acetyl-CoA is probably inequilibrium with a larger unlabelled endogenous pool than is acetoacetate], and (*c*) the low incorporation of C-2 compared with C-3 (C-2 becomes randomized and appears in both C-1 and C-5 of MVA and, as C-1 of MVA is lost during incorporation into terpenoids some of the label associated with C-2, but none of the C-3 label, is similarly lost).

Incorporation of CO_2 into carotenoids.—Coon and his group have shown that CO_2 is actively fixed in animal tissues during the metabolism of leucine [see (43)]. It has now been demonstrated that in *P. blakesleeanus*, $^{14}CO_2$ is fixed into β-carotene in the presence but not in the absence of leucine (13, 14, 23). This observation can be accommodated in the scheme already given in Figure 4. If C-1 and C-5 of HMG-CoA were not randomized by reactions

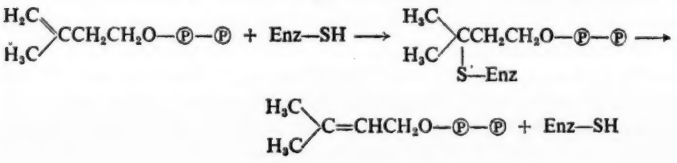
(B) and (C), all the CO_2 fixed in reaction (A) would become C-1 of MVA and thus inevitably be lost when MVA was built up into the β-carotene molecule.

There is a significant incorporation of $^{14}CO_2$ into β-carotene by isolated tomato slices, the amount incorporated being considerably greater than when [^{14}C]-acetate is the substrate (12, 13, 54); on the other hand, carrot root slices, which actively incorporate labelled acetate, fail to incorporate $^{14}CO_2$ (34). The causes of these differences have not yet been investigated.

CO_2 is by far the most efficient precursor of carotenoids in illuminated etiolated maize seedlings which are rapidly synthesizing plastid carotenoids (55, 56). Neither [2-^{14}C]-acetate nor [2-^{14}C]-mevalonate can penetrate the root system of intact maize seedlings, but excised seedlings rapidly incorporate both substrates from an aqueous solution into the unsaponifiable matter; however, the label is only insignificantly incorporated into the β-carotene synthesized on illumination. In contrast, $^{14}CO_2$ under the same conditions is preferentially incorporated into the β-carotene synthesized in the chloroplasts formed on illuminating intact or excised etiolated seedlings. The failure of acetate and mevalonate to be effectively incorporated [which confirms earlier experience with excised leaves using [^{14}C]-acetate (57), and with maize seedlings using [^{14}C]-glutamate (58)] is almost certainly due to their failure to reach the site of carotenoid synthesis rather than to the existence of a novel pathway of biosynthesis. As most of the sterols that become labelled under these conditions are located in the chloroplasts, the question arises as to why they, but not carotenoids, are labelled. The reason is not known, but three possibilities exist: (*a*) acetate and mevalonate cannot enter the chloroplasts but are converted into sterols in the stroma and the sterols are then transferred to the chloroplasts; (*b*) the substrates can enter the chloroplast, but are rapidly taken up by the sterol-synthesizing system before they reach the site of carotenoid synthesis; and (*c*) the channelling of substrates into either the steroid or carotenoid pathway is controlled metabolically; for example, possibly by the relative availability of TPN and DPN.

Polymerization of Isopentenyl Pyrophosphate (IPP)

This aspect of terpenoid biochemistry has been studied in yeast extracts by Lynen and his colleagues (41, 59) with special reference to the 15-C unit which dimerizes to form squalene, the acyclic steroid precursor. Two enzymes have been obtained, the first, IPP isomerase, converts IPP into β,β-dimethylallyl pyrophosphate, which acts as the starter for polymer growth. The probable mechanism for the reaction is given in Scheme 6. In the presence of the



Scheme 6.

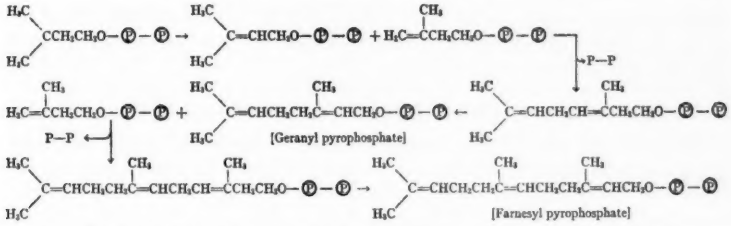
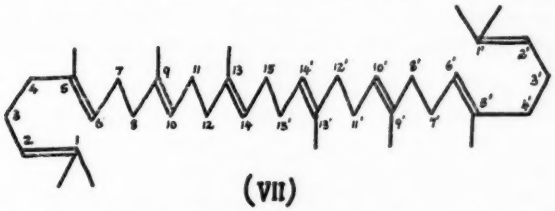


FIG. 5. Postulated mechanism for the formation of farnesyl pyrophosphate. (the 15-C precursor of squalene) from IPP.

second enzyme, one molecule of β,β-dimethylallyl pyrophosphate condenses with one molecule of IPP with the liberation of pyrophosphate to form geranyl pyrophosphate. This, in turn, condenses with a further molecule of IPP to yield farnesyl pyrophosphate (Fig. 5). This is the compound which dimerizes "tail to tail" to yield squalene. The exact mechanism of dimerization is not known but a highly plausible scheme has been put forward by Popják and his colleagues (60). If the reactions indicated in Figure 5 were carried one step further, then the resultant would be the 20-C compound, geranylgeranyl pyrophosphate, which, by analogy with squalene synthesis, would be the dimerizing unit for the 40-C carotenoids. The formation of such a compound has not yet been unequivocally demonstrated, but acid-labile derivatives of geranylgeraniol have been detected on incubating farnesyl pyrophosphate and IPP in the presence of a yeast extract (41).

THE 40-C PRECURSORS OF CAROTENOIDS

By analogy with the formation of squalene as the 30-C precursor of sterols we can assume that (VII) (7, 8, 11, 12, 15, 7′, 8′, 11′, 12′, 15′-decahydrolycopene) the 40-C analogue of squalene, represents the immediate



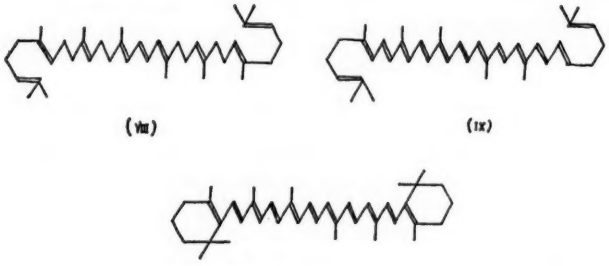
(VII)

precursor of all carotenoids; this compound has not been detected, but the analytical techniques currently in use may not be adequate. Stanier (61), however, feels that phytoene (7,8,11,12,7′8′,11′,12′-octahydrolycopene) (VIII) is the logical initial 40-C precursor, and there are arguments in its favour, perhaps the most cogent being that the existence of the central double bond would give rigidity to the molecule and prevent the widespread cyclization noted in the steroids and other triterpenoids. However, the queries which naturally arise from either assumption are: (*a*) What are the intermediates between (VII) or (VIII) and lycopene (IX)? (*b*) At what stage

does cyclization occur to produce carotenoids such as β-carotene (X)? (*c*) How and when are the oxygen functions inserted into xanthophylls? and (*d*) How do *cis-trans* isomers arise?

Intermediates in the Production of Lycopene

To Porter & Lincoln (62), the existence of a number of partly saturated lycopene derivatives as minor polyene components of commercial tomatoes



(VIII) (IX)

(X)

and as major components of certain tomato crosses suggested that they were intermediates in the formation of lycopene. This scheme, somewhat modified from the original, is indicated in Figure 6. Although the chemical structures of some of the intermediates have not been unequivocally demonstrated (see 63), they are very likely to be those indicated. This being so, the conversion of (VII) into lycopene involves: firstly, removal of 2 hydrogen atoms (VII→phytoene), secondly, two isomerizations (phytoene→phytofluene →ζ-carotene), and finally, two dehydrogenations (ζ-carotene→neurosporene →lycopene). Both of these last steps involve the removal of four hydrogen atoms and on general biochemical grounds one would expect each of these reactions to take place in two stages involving the stepwise removal of two

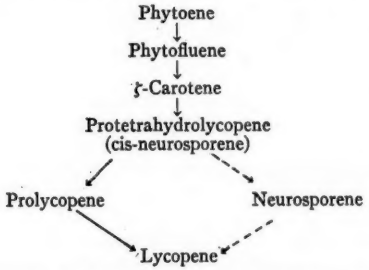
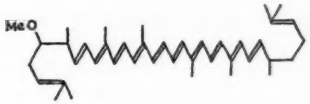


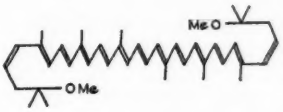
Fig. 6. Postulated mechanism for the formation of lycopene from a partly saturated precursor.

hydrogen atoms. So far no intermediates have been reported, but a possible intermediate between ζ-carotene and neurosporene is 'Carotene *X*' or P412 (64, 65). Other genetic support for the sequence outline in Figure 6 arises from work on various mutants of the photosynthetic bacterium *Rhodopseudomonas spheroides* which under anaerobic conditions synthesizes mainly pigment *Y* (XI) (66). A blue-green mutant accumulates phytoene, a green mutant ac-



(XI)

cumulates neurorporene with traces of phytofluene and ζ-carotene, and a brown mutant accumulates *Y* and large amounts of neurosporene (67, 68). Other support comes from experiments using the compound diphenylamine (DPA) which specifically inhibits carotenogenesis in a number of microorganisms [see (36)]; the inhibition is accompanied by accumulation of the phytoene series. For example, the photosynthetic bacterium *Rhodospirillum rubrum*, which normally synthesizes the lycopene derivative spirilloxanthin (XII), in the presence of DPA will channel synthesis into the phytoene series and inhibit spirilloxanthin synthesis (69); if the cells are washed free from DPA, resuspended in phosphate buffer and illuminated, then spirilloxanthin is rapidly synthesized. A kinetic study of the synthesis of spirilloxanthin



(XII)

under these conditions indicated that lycopene (and eventually spirilloxanthin) was produced sequentially from phytoene via the series indicated in Figure 6 (65). Furthermore, the specific activities of the phytoene series, compared with that of lycopene in ripening tomatoes injected with ^{14}C-mevalonate, are not inconsistent with the sequential dehydrogenation hypothesis (25). An earlier conclusion to the contrary (38) was based on results obtained with incompletely purified polyenes. It is essential in isotope work on carotenogenesis either to crystallize the polyenes or to make pure derivatives; "chromatographically pure" polyenes are not isotopically pure, for they are often accompanied by traces of highly active, as yet unidentified compounds.

Cyclization of the 40-C Precursor

The available evidence, although indirect, suggests that in most cases cyclization occurs before complete unsaturation, i.e., lycopene is not the precursor of β-carotene. Two distinct pathways are indicated in normal tomatoes from studies on the effect of ripening temperature on pigment synthesis. At 30° C. or above, the synthesis of lycopene and the phytoene series is inhibited whilst that of β-carotene is essentially unaffected (70, 71). However, it is possible under certain circumstances to channel lycopene or its precursors into β-carotene synthesis in tomatoes. This was observed in an orange phenotype obtained by back-crossing a *Lycopersicon esculentum* × *L. hirsutum* hybrid to *L. esculentum*. In this phenotype lycopene is replaced by an equivalent amount of β-carotene and the change is ascribed to a single dominant gene *B* (72 , 73). Furthermore, the synthesis of β-carotene in the orange phenotype is temperature-sensitive (74), which is, as just stated, a characteristic of lycopene synthesis in the wild strain. It is not yet possible to decide whether *B* achieves this effect by controlling ring closure of lycopene or by channelling a common early precursor from the lycopene pathway into the β-carotene pathway. The fact that the F_1 generation of high β-carotene fruit contains more γ-carotene (XIII) (a possible intermediate be-

(XIII)

tween lycopene and β-carotene) than do the high lycopene strains, provides some support for the idea that lycopene is converted into β-carotene.

A pink mutant of *Corynebacterium michiganese*, produced by exposure of the wild strain to either uranium sulphate or 5-aminoacenaphthene, produces only acyclic carotenoids (spirilloxanthin and lycopene), an orange strain produces only cyclic carotenoids [β-carotene, canthaxanthin (4,4′-diketo-β-carotene) and cryptoxanthin (3-hydroxy-β-carotene)], whilst the wild strain (yellow) produces both types (lycopene and cryptoxanthin) (75). These observations, combined with the finding that a red back-mutant produces only lycopene, suggests that there may be independent genetic control for the production of acyclic and cyclic carotenoids, and supports the idea that the bifurcation of the two pathways occurs early in the biosynthetic chain. Further support for this view comes from experiments on the effect of DPA on β-carotene synthesis in *P. blakesleeanus*. If mycelia grown in the presence of DPA and containing little or no β-carotene but large amounts of the phytoene series (76) are washed free from DPA and resuspended in a medium containing glucose but no nitrogen, β-carotene is rapidly synthesized but not at the expense of the phytoene series. When DPA cultures are prepared in the presence of [2-^{14}C]-mevalonate, the phytoene series is strongly

labelled and resuspension of the washed cells in unlabelled glucose yielded labelled β-carotene (13); thus the β-carotene must have been formed to some extent from endogenous precursors which are not the phytoene series. These observations can be reconciled with those on *R. rubrum*, discussed above, if it is assumed that cyclization of a C-40 precursor is an early step in the pathway of β-carotene synthesis, and that DPA not only inhibits dehydrogenation of saturated cyclic and acyclic precursors but also stimulates synthesis of the phytoene series. Evidence in favour of two separate functions of DPA include: (*a*) massive amounts of phytoene always accumulate in the presence of DPA in both *P. blakesleeanus* (76) and *R. rubrum* (61); and (*b*) in the presence of adenosine-5′-phosphate (AMP), *P. blakesleeanus* produces the phytoene series in large amounts without any concomitant inhibition of β-carotene synthesis (77). The situation with respect to DPA can then be

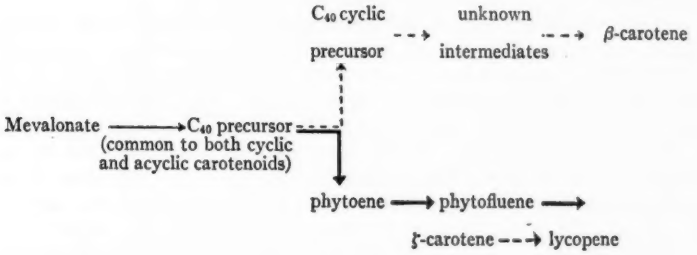


FIG. 7. Postulated mechanism of action of DPA on carotenogenesis.
– – – – reactions inhibited by DPA
——— reactions stimulated by DPA
It is assumed that once the pathways branch there is no further interconversion.

envisaged as in Figure 7. *P. blakesleeanus* grown in the presence of DPA accumulates a 40-C precursor probably common to both cyclic and acyclic carotenoids, and stimulates synthesis of the phytoene series. When DPA is removed, the C-40 precursor cyclizes and is eventually converted into β-carotene whilst the phytoene series are not altered because (*a*) they cannot be converted into lycopene in significant amounts, as *P. blakesleeanus* has only a very limited ability to synthesize lycopene (78), and (*b*) they are beyond the point of no return as far as cyclization is concerned and thus cannot be channelled into β-carotene synthesis. The common 40-C precursor may well accumulate in the phytoene fraction, because using the analytical techniques presently available, it would be difficult to separate almost completely saturated polyenes [see, e.g. (25)]. Furthermore, the sequential dehydrogenation of the cyclized 40-C precursor might give rise to early intermediates not containing more than three conjugated double bonds, and thus would not be detected by the conventional methods of carotenoid analysis.

In contrast to *P. blakesleeanus*, *R. rubrum* is predisposed to produce acyclic carotenoids so that when DPA is removed from an inhibited culture, the phytoene series are, under suitable conditions, rapidly converted into spirilloxanthin. This conversion is probably facilitated by the members of the phytoene series being present in the chromatophores, which appear to be the site of their conversion into spirilloxanthin.

Results that at first sight appear contrary to these views are those of Claes (79) on a mutant (5/520) of *Chlorella vulgaris* which in the dark synthesizes some members of the phytoene series; anaerobic illumination of dark grown cells results in the synthesis of β-carotene and an unidentified pigment *X* with the simultaneous disappearance of the members of the phytoene series. Thus it would appear that β-carotene was arising from the phytoene series; however, the reported data will permit the alternative interpretation that the phytoene series is being converted into *X* (which appears to be acyclic) and that β-carotene may be arising from another source.

If one now turns to the mechanism of cyclization involved in the production of carotenoids such as β-carotene, one finds only complete ignorance. All that can be said is that it cannot be the same as that operating for sterols and triterpenoids in general. The first product of squalene cyclization in steroid biosynthesis is lanosterol, formed as indicated in Figure 8 and containing an hydroxyl group; the hydroxyl group is present because the cyclization is initiated by OH^+ attack [see (43)]. All the evidence in the carotenoid field is that oxygen-containing pigments (xanthophylls) are produced from the fully unsaturated hydrocarbon carotenes (see below).

The problem of how cyclic carotene isomers, i.e., α-, β-, and γ-carotenes, are formed is also unsolved.

Insertion of the Oxygen Function into Carotenoids

Hydroxylated carotenes.—In higher plants no existing evidence indicates how the major chloroplast xanthophyll, lutein (3,3′-dihydroxy-α-carotene), is formed. There is not even an indication as to whether it arises *de novo* or by hydroxylation of the corresponding carotene. However, we do have good evidence that in green algae the insertion of oxygen occurs as the last step in the biosynthetic chain. It has already been noted that mutant 5/520 of *C. vulgaris* synthesizes the phytoene series in the dark and that anaerobic illumination of dark-grown cultures results in the synthesis of α- and β-carotenes and pigment *X*. If these cultures are now returned to the dark and allowed access to oxygen xanthophylls are formed and there is a simultaneous disappearance of the carotene fraction (79). These results indicate that the carotenes are the immediate precursors of the xanthophylls and that the overall reaction can be summarized as in Scheme 7.

$$\text{Unsaturated polyenes} \xrightarrow[\text{anaerobically}]{\text{light}} \text{Carotenes} \xrightarrow[\text{aerobically}]{\text{dark}} \text{Xanthophylls}$$

Scheme 7.

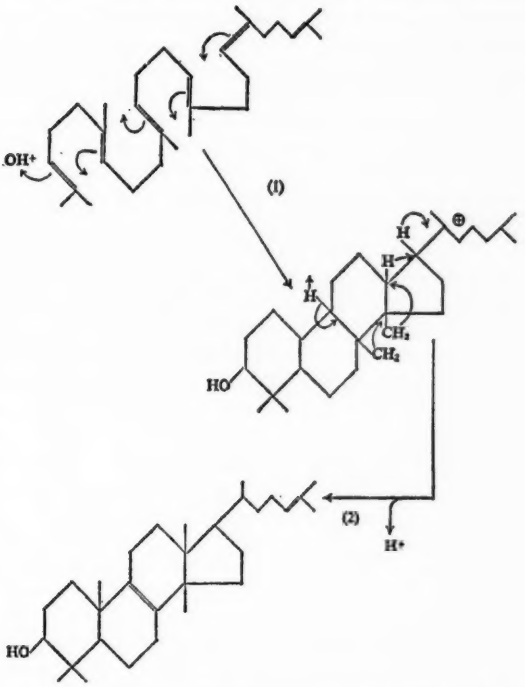
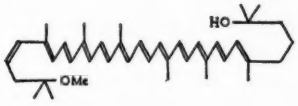


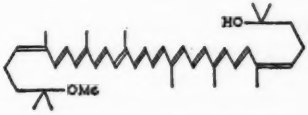
FIG. 8. Probable mechanism for cyclization of squalene to lanosterol.

Isotope experiments with a deep red strain of *Mycobacterium phlei* indicate that myxoxanthophyll, the main xanthophyll present (80), is formed from hydrocarbon precursors, but it is not yet certain that the carotenes in the hydrocarbon fraction are the active constituents of this fraction (81).

Methoxylated carotenoids.—A characteristic of the purple bacteria is the synthesis of methoxylated carotenoids such as spirilloxanthin (XII), which is the main pigment of mature *R. rubrum* cultures (82). In exponentially growing cells, however, the situation is different and lycopene (IX), rhodopin (1-hydroxylycopene), P-481 (1-methoxy-1,2-dihydro-3,4-dehydrolycopene),



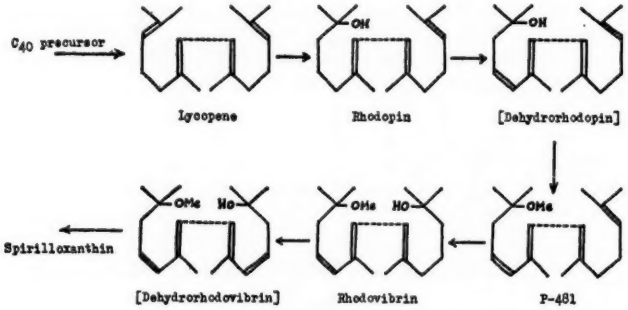
(XIV)



(XV)

and rhodovibrin (hydroxy-P-481, either XIV or XV) are the main pigments, with spirilloxanthin present only in traces. It was originally suggested that rhodopin was identical with lycoxanthin (3-hydroxylycopene) (83) but recent investigations indicate that the hydroxyl group is in position 1 (84). The structure of P-481, a pigment first observed in the purple sulphur bacterium *Chromatium* sp. (83) has been derived by Barber *et al.* (85) and Jensen (86). The two possible structures indicated for rhodovibrin have been proposed by Jensen (87); they differ from that suggested by Barber *et al.* (85).

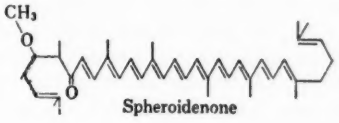
If washed young cells of *R. rubrum* are resuspended in phosphate buffer anaerobically in the light, then there is a marked synthesis of spirilloxanthin accompanied by a concomitant disappearance of the original pigments, but there is no net synthesis of pigment (65, 88). Our present knowledge of the structure of the various pigments allows a formulation of the probable stages in the conversion of lycopene into spirilloxanthin to be proposed (Scheme 8). Undetected postulated intermediates are enclosed in square brackets. This scheme requires the insertion of a 1-C (methyl) group after the synthesis of



SCHEME 8.

the hydroxyl derivatives. Direct support for this comes from the demonstration that young washed cells of *R. rubrum* incubated anaerobically in phosphate buffer in the light and in the presence of [^{14}C]-formate incorporate the label specifically into the methoxyl group of spirilloxanthin (89).

Keto-carotenoids.—Anaerobic cultures of the nonsulphur photosynthetic bacterium *Rhodopseudomonas spheroides* are yellowish-brown, but rapidly turn purplish-red on exposure to oxygen (90, 91). The colour change is due essentially to the conversion of the yellow pigment (*Y*) (XI) into the purple pigment *R* (spheroidenone) (XVI), by the insertion of a carbonyl group at one end of a polyene chain (66, 90, 91, 92). The reaction is not reversible, has not yet been carried out in cell-free systems, and is not susceptible to most common inhibitors of oxidation, except azide at pH 4.8 but not at 6.8



Spheroidenone

(XVI)

The mechanism of the oxidation is not yet clear, but a possible mechanism is one based on the similarity of the reaction to that catalysed by tryptophan pyrrolase [see (43)].

The massive and sudden extraplastidic synthesis of astaxanthin (3,3′-dihydroxy-4,4′-diketo-β-carotene) in encysting *Haematococcus pluvialis* is not at the expense of the plastid carotenoids, and no intermediates in its formation have yet been detected (93).

It has been suggested from studies on the carotenoids of ripening *Capsicum annuum* fruit that capsanthin and capsorubin arise from zeaxanthin (3,3′-dihydroxy-β-carotene) via antheraxanthin (5,6-epoxyzeaxanthin) and violaxanthin (5,6,5′,6′-diepoxyzeaxanthin) (94). From the recently elucidated structure of capsanthin and capsorubin (p. 219) it is clear that a pinacolic rearrangement of the epoxides could give rise to these pigments (2).

Carotenoid epoxides.—Epoxides of β-carotene, which are only minor components of fresh green leaves, rapidly accumulate at the expense of β-carotene in excised tomato leaves maintained in the dark and in the absence of CO_2 (57). This observation suggests that the epoxides are formed directly from the hydrocarbons and, as epoxides soon disappear from leaves allowed to photosynthesize, whilst β-carotene synthesis begins only after a lag period of about 24 hr., it would seem that epoxides are the first steps in the pathway of degradative oxidation of carotenoids in plant tissues.

It has also been reported that in excised leaves kept aerobically in the dark an increase in violaxanthin occurs with an accompanying loss of an equivalent amount of lutein (95). Unlike the situation with the carotenes, removal of leaves into the light reverses the situation. An intriguing aspect of this report is that apparently a β-carotene derivative (violaxanthin) and an α-carotene derivative (lutein) are freely interconvertible.

Recently it has been found that the cotyledons of etiolated French beans are relatively rich in carotenoid epoxides which do not occur to a significant extent in the true leaves (96). The reason for this differential distribution is not yet known.

The carotenoid distribution in flower petals is characterized by the wide occurrence of epoxides, often as the major pigments (see 97). These pigments may also be formed by "epoxidation" of carotenes and xanthophylls, but no experimental evidence is yet available on this point.

Formation of cis-isomers.—Many plants produce various *cis*-isomers,

especially in their fruit (see 97), but the mechanism involved has been examined only in two instances. The *Chlorella* mutant 5/520 of Claes, which has already been discussed in previous sections, produces various poly-*cis*-isomers of tetrahydrolycopene (neurosporene) and lycopene in the dark (79, 98). On illuminating the cells aerobically with blue light and anaerobically with red light the pigments isomerize to all-*trans*-neurosporene and -lycopene respectively (98, 99). It was assumed that the blue light was absorbed directly by the carotenoid pigments, whilst the red light was absorbed by the traces of chlorophyll present in the cells and the energy then transferred to the carotenoids. This view has been confirmed by experiments with pure solutions in light petroleum of poly-*cis*-neurosporene (proneurosporene) and chlorophyll-*a*; irradiation with red light of a solution of proneurosporene produces little isomerization whereas in the presence of chlorophyll-*a* and in an atmosphere of N_2, conversion to the all-*trans* form is almost complete; oxygen inhibits the isomerization (99). A possible relationship exists between this work and an earlier report that flower petals of *Mimulus longiflorus* developed in diffuse light on excised budded stems contained considerable amounts of poly-*cis*-lycopenes and poly-*cis*-γ-carotenes, whereas petals developed on the plant contained no *cis*-isomers (100). These intriguing observations raise the possibility that *cis*-isomers are normal precursors of all-*trans* carotenoids. Furthermore, it should be emphasized that naturally occurring phytofluene is a *cis*-isomer (101).

A study of various tomato genotypes synthesizing different carotenoids [see (43) for full details] has revealed the existence of gene *T* which controls the channelling of precursors into either lycopene (*T*) or ζ-carotene and prolycopene (poly-*cis*-lycopene) (*t*). It is possible that *T* exerts its control in one of two ways: either by stereochemical guidance, the conversion of prolycopene into lycopene (102); or by controlling dehydrogenation, the conversion of ζ-carotene into lycopene, possibly *via* prolycopene (62). Further studies combining biochemistry and genetics are urgently required in this field.

Carotenoid Formation in Cell-Free Systems

In order to determine the details of the pathway of carotenoid synthesis, it is necessary to develop a purified enzyme system which will carry out the synthesis. A start has recently been made on this problem. A cell-free system has been obtained from carrot root slices which incorporates both [^{14}C]-acetate and [^{14}C]-mevalonate into β-carotene (14, 55, 63). A similar system from tomato parenchyma incorporated [^{14}C]-mevalonote but not [^{14}C]-acetate into lycopene (103); ATP, DPN, TPN, glutathione, Mn^{2+}, and O_2 are necessary for maximal activity. In both these preparations the incorporation of mevalonate is of the order of 0.04 per cent. Recently, however, Modi & Patwa (104) have reported a net synthesis of β-carotene by extracts of *P. blakesleeanus* and Yokoyama *et al.* (105) have prepared an extract of the same organism which incorporates 1 to 4 per cent of added [2-^{14}C]-mevalonate into β-carotene. The co-factors required for this system are ATP, DPNH, TPNH, TPN, Mn^{2+}, Cu^{2+}, and O_2 (H. Yokoyama, personal communication). Isolated

chromatophores of *R. rubrum* incorporate [2-^{14}C]-acetate and [2-^{14}C]-mevalonate into spirilloxanthin, the latter substrate being relatively much more effective (37). All these systems synthesize carotenoids as well as steroids (or related compounds) and to date no report has been published of the separation of the steroid-synthesizing system from the carotenoid-synthesizing system. However, an important observation in this context is that addition of DPN to the complete system of Yokoyama *et al.* channels activity into steroids rather than carotenoids, whilst in the absence of DPN β-carotene synthesis is preferred (H. Yokoyama, personal communication).

FUNCTION OF CAROTENOIDS IN PLANTS

Because of limitations of space it has only been possible to discuss the function of carotenoids in photosynthesis, photophosphorylation and photosensitization. For details on other aspects see (36).

Photosynthesis

It is now common knowledge that the photosynthetically active units of higher plants and protists invariably contain carotenoids alongside chlorophylls (43, 106); it is also clear that carotenoids cannot play an initiating role in photosynthesis because many nonchlorophyllous tissues rich in carotenoids are known (e.g., carrot roots) but none of these can photosynthesize. Furthermore, a pale-green mutant of *Chlamydomonas*, which is almost completely deficient in carotenoids and low in chlorophylls, is capable of photosynthesis (107). Measurements of the action spectrum for photosynthesis in algae have shown that the light absorbed by the constituent carotenoids are photosynthetically active in three classes (Chlorophyceae, Phaeophyceae, Bacillariophyceae) (108, 109). However, in those red algae (Rhodophyceae) and blue-green algae (Cyanophyceae) so far examined, carotenoid-absorbed light is only very slightly effective, if at all (109). The reasons for these differences are not yet apparent, because as in all other photosynthetic organisms the carotenoids are firmly located in the chloroplasts. Direct measurements of action spectra in green leaves are difficult to make but the action spectrum for the Hill reaction in isolated chloroplasts indicates that carotenoid-absorbed light is effective in promoting this reaction (110).

The participation of carotenoids in photosynthesis in the photosynthetic bacterium *Rhodospirillum rubrum* has been clearly demonstrated, both in young and mature cultures in which different carotenoids are present (see p. 233) (111, 112). The pigments are also active in *R. molischianum*, *Rhodopseudomonas spheroides*, and *Chromatium* spp., but there is as yet no information regarding their activity in the green sulphur bacteria (e.g., *Chlorobium* spp.) (113, 114). The effect is still apparent in isolated chromatophores, and is unaffected by changes in osmotic pressure or ionic strength of the medium in which the chromatophores are isolated (119). The efficiency of the energy transfer from carotenoids to chlorophylls is nearly 100 per cent for diatoms and *Chlorella* (113), but only 30 to 40 per cent for all the photosynthetic bacteria examined except *Rhodopseudomonas spheroides*, in which the effi-

ciency was about 90 per cent (114). Removal of a major part of the carotenoids from the chromatophores of *R. rubrum* does not result in an increased efficiency of the remaining fraction. Thus it appears unlikely that the spirilloxanthin is present at two sites in the chromatophore, one of which is inactive whilst the other is 100 per cent efficient (114).

The mechanism probably involved in the energy transfer is that of "internal" or "inductive resonance." It would appear that in most of the photosynthetic units a primary requirement for inductive resonance—that the participating pigments must be located together within a wave zone which is small compared with the wavelength of the exciting light—is fulfilled. This condition is not fulfilled in solutions of carotenoids and chlorophylls in organic solvents, but it can be fulfilled by dispersing the pigments in detergent micelles to give a local pigment concentration of about 0.1 *M* (115); under these conditions, but not in simple solutions, carotenoid-absorbed light will stimulate chlorophyll fluorescence. A second requirement for inductive resonance is that the fluorescence band of the primary light absorber should overlap the absorption band of the acceptor pigment. This requirement is known to exist for the phycobilins, but as yet carotenoid fluorescence has not been demonstrated, although indirect evidence suggests that fucoxanthin fluorescence exists in the region above 600 mμ (115).

Some of the last investigations of the late R. Emerson indicate that carotenoids may have a more subtle effect than merely extending the effective spectral range for photosynthesis. He found that in algae, light absorbed by chlorophyll-*a* is only effective when the organisms are simultaneously excited with light of a shorter wavelength (116, 117). However, this effect can also be produced by pigments other than carotenoids.

Photophosphorylation

The action spectrum for photophosphorylation in spinach chloroplasts strongly suggests that the carotenoid-absorbed light is used in promoting phosphorylation (118). In the purple bacteria, on the other hand, the light absorbed by carotenoids is only slightly effective, having much less than the 50 per cent efficiency noted in energy-transfer experiments (119); furthermore, the variation in the osmotic strength of the suspending medium causes changes in the efficiency of the carotenoid-absorbed light (120). Carotenoid-free chromatophores of *Chromatium* spp., prepared by culturing the bacterium in the presence of DPA, will photophosphorylate normally under anaerobic conditions (121), suggesting that carotenoids are not essential for this key reaction in photosynthesis. Furthermore, assuming that the steps revealed by the Hill reaction are essential to photophosphorylation, then, again, carotenoids can be ruled out of this reaction; extraction of lyophilized chloroplasts with cold light petroleum almost completely abolishes the ability of the chloroplasts to carry out the Hill reaction (122). Addition of pure β-carotene to this preparation has little activating effect whilst vitamin K preparations are extremely active (123).

Protection Against Photosensitivity

A series of investigations by Stanier and his colleagues has revealed a function of carotenoids in photosynthetic tissues that may not be correlated with their colour (i.e., their ability to absorb visible light) but may be of more fundamental importance than the functions already discussed. A blue-green mutant of *Rhodopseudomonas spheroides* in which synthesis of coloured carotenoids has been eliminated but in which phytoene synthesis is known to occur will grow photosynthetically under anaerobic conditions; however, in the presence of both light and oxygen it is rapidly killed (124, 125); neither agent is lethal alone. The photo-receptor for photosensitization is bacteriochlorophyll. If the reaction is carried out at 0–5°C. the bacteriochlorophyll remains in the dead cells (126); at higher temperatures side reactions occur and the bacteriochlorophyll is destroyed (124). It was concluded from these experiments that carotenoids protected the organism against photosensitization. The weakness in this argument was that the mutant could have undergone other unrecognized mutations and possibly no causal relationship existed between the absence of carotenoids and photosensitivity. This uncertainty has now been removed; when carotenoid synthesis in normal *Rhodospirillum rubrum* is suppressed by diphenylamine the resulting cells are photosensitive under aerobic conditions. Furthermore, if the cells are washed free from DPA and allowed to synthesize small amounts of carotenoids, they cease to be photosensitive (127). The extent of the protection appears to be a function of the length of the conjugated chain in the constituent carotenoids; a green mutant of *R. spheroides*, containing mainly neurosporene, becomes photosensitive under high light intensities whilst the wild strain shows no such effect under the same conditions (61).

The elucidation of the views just expressed were carried out using photosynthetic bacteria which never liberate oxygen during the photosynthetic act. In higher plants and algae, which are oxygen-evolving phototrophs, removal of carotenoids should prevent photosynthetic development or, if oxygen is excluded, its production during photosynthesis would lead to photosensitization and death (61). In support of this view we note that the various carotenoid-less or carotenoid-deficient mutants of *Chlorella* produced by Claes (see p. 232) are photosensitive, as is a carotenoid-deficient mutant of *Chlamydomonas*. Aerobic exposure of *Chlorella* 9a to high light intensities results in a rapid uptake of oxygen and the simultaneous destruction of the chloroplast pigments; pigment loss is prevented by maintaining the cells under anaerobic conditions during illumination (128). However, in contrast to the situation in the photosynthetic bacteria, blue (carotenoid-absorbed) light destroys the pigments whilst red (chlorophyll-absorbed) light is without effect.

Two observations militate against the acceptance of protection against photosensitization as a universal function of carotenoids in photosynthetic organisms: (*a*) the photosynthetic bacteria *Chromatium* spp. and *Chlorobium* spp., which contain carotenoids, are obligate anaerobes; and (*b*) carotenoid-

deficient mutants of higher plants are not killed by aerobic exposure to light. The first objection has not yet been answered, but it is suggested that the second is due to the difference in cell structure between higher plants and protists (61). Assuming that photooxidation causes death by destruction of DNA, then the most efficient effect should be observed in cells in which the nucleus and the photosynthetic apparatus lie closely together. Electron microscopy has revealed that the nuclear region of the photosynthetic bacteria is surrounded by numerous chromatophores which occupy a large volume of the cytoplasm (129); furthermore, this view has been generally confirmed by later work (130, 131, 132). In *Chlorella* much of the cell volume is taken up by the chloroplasts. Conversely, in the cells of higher plants there is a greater degree of separation between the nucleus and the chloroplasts.

The mechanism by which carotenoids exert their protective effect is not known, but Stanier (61) favours the view that "assuming excited chlorophyll molecules always have a certain probability of carrying out photooxidations, the carotenoids could be envisaged as providing reactive and readily available substrates, through the oxidation of which damage to chlorophyll itself or to other cell constituents is minimized." The experiments of Smith & Ramirez (133, 134) are quoted in support of this view; these investigators discovered that if *R. spheroides* changes from an aerobic to anaerobic state in the dark, or if anaerobic dark cultures are illuminated, peaks appear in the difference spectrum at 445, 475, and 508–509 mμ and troughs appear at 490 and 525 mμ. These variations were taken to indicate a decrease in the absorption peaks of the normal carotenoids and the appearance of absorption peaks at longer wavelengths. These changes are reversible and appear not to be connected with the irreversible $Y \rightarrow R$ change described on p. 234. Similar changes are observed with *R. rubrum*, although it will be recalled that the carotenoid components of the two bacteria are quite distinct. It should be pointed out with reference to the present problem, however, that Smith & Ramirez do not consider the reported changes to be oxidation-reduction changes but as caused by a change in the structure of the cell during the initiation of the electron-transfer processes with "a resultant perturbation of the carotenoid molecule."

The apparently fortuitous distribution of carotenoids in nonphotosynthetic micro-organisms has not yet been satisfactorily explained and an attempt to show that they exerted a protective action against photosensitization in *Corynebacterium poinsettiae* (135) was not successful. Colourless mutants of this organism and colourless cells of the native strain (produced by growing the cells in the presence of DPA) do not show photosensitization under high light intensity (4,000 foot-candles for 4 hr.). However, in the presence of toluidine blue, the sensitivity to light of the carotenoid-deficient cells was very marked compared with that of the native strain; presumably the colourless cells do not contain sufficient amounts of a naturally occurring pigment to prevent photosensitization.

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BIOCHEMISTRY OF SENESCENCE[1,2]

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Introduction

One problem of biology involves understanding the factors that control the timing of the various periods in the life cycle of a cell. The dramatic events of cell division and cell enlargement have received much attention but the gradual changes during maturation and the onset of cellular senescence are less easily observed and have received less attention. Nevertheless, all phases of the life cycle of a single cell or a group of cells are of equal intrinsic interest and a knowledge of each is of equal potential practical application. Starting from the point of view that postharvest fruits and vegetables are merely specific examples of maturing and senescing cells, it is the purpose of this review to consider the general subject of the metabolism of aging cells with particular reference to cells and tissues of higher plants.

General Theories of Aging

There seems no reason to doubt that senescence is a universal phenomenon in living organisms (1, 2, 3). The visible changes that occur during senescence in higher plants and animals are easily recognizable although not always readily susceptible to quantitative measurement. The physical changes occurring at the cellular and subcellular level have been described only briefly (1, 2, 4) and while observations of the metabolic changes that accompany aging are more numerous, they are disappointing in that cause and effect relationships are seldom clear. Lansing (2) has described aging as "a problem of cellular change—of unfavourable change in the ability of protoplasm to maintain itself." To make this description more explicit it is useful to recall Dixon's definition of living matter as "a system of unstable catalysts being kept in existence by the occurrence of the reactions which they catalyse" (5).

The suggestions that the life span of an individual is limited by the loss of optical purity of certain crucial substances (6), by increasing protein immobilization due to progressive cross-linking reactions (7, 8), or by thermal denaturation of irreplaceable molecules (9), are largely speculative and need no further discussion here.

In many respects the loss of cellular vitality in senescence resembles the effects of radiation injury (9). The similar effects of aging and of ionizing radiations on the ability of mice to withstand stress suggest that, in some

[1] The survey of literature pertaining to this review was concluded in October 1960.

[2] The following abbreviations are used: EMP (Embden-Meyerhof-Parnas respiratory pathway); NAA (naphthaleneacetic acid); 2,4-D (2,4-dichlorophenoxyacetic acid); 2,4,5-T (2,4,5-trichlorophenoxyacetic acid).

respects, ionizing radiation accelerates the normal process of aging (10, 11). However, because massive application of nitrogen mustard does not induce premature aging, Curtis & Gebhard (11) believe that mutation in somatic cells does not cause the effects observed. During the germination of wheat and soya bean, maximum sensitivity to ionizing radiation occurs at the time the growth substance in the endosperm is mobilized. However, the cells of leaves are remarkably resistant to radiation; a dose of 40,000r is without effect (12). Neary (13) has suggested that there is "some intrinsic process of biological aging, enhanced by radiation, which operates from the start of life and is virtually complete by the time the changes of old age begin to appear." This theory "assumes that there is a common process at work in natural and radiation-induced aging which is fundamental, inevitable and irreversible." Once aging has been induced its development proceeds autonomously and autocatalytically (13). Although there is no conclusive evidence to indicate that loss of nuclear function by a cell is a primary event in senescence, there is no doubt that the nucleus of a somatic cell exerts a profound effect on the metabolism of the rest of the cell (14) and that, conversely, events in the cytoplasm have a profound effect on the nucleus (14 to 17).

The accumulation with age of some inhibitor or deleterious substance has frequently been proposed as a cause of senescence (18, 19). Lansing's elegant experiments (18) on factors affecting the life span of rotifers led him to suggest that a transmissible, cumulative, reversible factor is responsible for aging in this organism. The accumulation and deposition of calcium salts in aging cells of plants and animals is well documented (20, 21). That this accumulation of calcium is to some extent a causative agent in the aging of rotifers is shown by the fact that a low level of calcium in the culture medium was associated with an increased life span; also, the experimental removal of calcium from rotifers with sodium citrate increased their life span by 50 per cent (22). Because calcium in old cells of *Elodea*, a rotifer, a planarian, and a toad tended to deposit in the cell cortex, Lansing suggested that cell permeability was being adversely affected (22). It has often been proposed that permeability changes are causative factors in the aging of plants (23, 24, 25) and animals (21), and recently such proposals have been vigorously restated to be of significance in senescent plant cells with auxin exerting a decisive influence (26).

De Kock and co-workers have studied responses of old and young leaves (27) and of tissues of old and young rats (28) to various metabolic inhibitors, and have found that the magnitude of response produced by the various inhibitors was less in the older tissues of both plants and animals. From these experiments they conclude that "the balance between the various processes changes with aging of the organism" and "that no metabolic system is lost or new system introduced during the life of the organisms" (28).

Over 50 years ago Rubner (29) proposed that an inverse relationship existed between rate of metabolism and duration of life (of animals) and that

various mammals during their lifetime used up approximately the same number of calories per unit weight. This theory gains much support from the many reports demonstrating that the life span of animals can be increased by lowering the body temperature [see the review by Bourliere (1)]. It is indisputable, of course, that the life span of postharvest plant tissues can be extended by judiciously lowering the temperature. It is interesting to consider Rubner's theory in terms of what is now known of the cellular components involved in energy metabolism. One attractively simple hypothesis is that the mitochondria of any cell have a definite total work capacity and senescence is the result of the failure of the mitochondria to be reproduced or repaired.

Development of this hypothesis depends upon gaining further knowledge of the biogenesis and life span of mitochondria. At the moment, any cell component (particulate or soluble, enzyme or coenzyme) required in the chain of reactions producing energy is a possible candidate for the role of limiting factor in total energy production. The influence of temperature on a life span can also be cited as support for other theories of aging. For example, a decrease in temperature would greatly reduce thermal denaturation of macromolecules. Measurement of the temperature coefficients of the induction and development of aging would be most useful as a means for elucidating possible mechanisms.

The foregoing theories and ideas have been presented without regard to their immediate applicability to the specific problems of postharvest physiology. In the following section the discussion will be limited to data and theories that deal almost entirely with plant cells and are more closely related to postharvest problems.

Respiration and Cellular Work During Senescence

Biale's contention, in his 1950 review of the postharvest physiology and biochemistry of fruit (30), that emphasis should be placed on respiration as a central process common to all fruits has been amply justified. It now seems satisfactorily established that, up to the time of the climacteric peak, respiration as measured by oxygen uptake is a reflection of the work performed by the cells, and most of this work is mediated by ATP. The individual papers that allow these conclusions have been reviewed recently in several excellent articles (30 to 34). It is also established that the mitochondria of postclimacteric (senescent) cells are still capable of oxidative phosphorylation and are still performing anabolic functions such as the synthesis of proteins and of pigments (35). Further, it is certain that normal ripening, in tomatoes at least, requires the expenditure of energy from oxidative phosphorylation because infiltration of whole, green, mature tomato fruit with dinitrophenol prevents the chemical and physical changes associated with ripening (36, 37). This requirement for energy for normal ripening is probably a general one because decreased oxygen tension also delays or prevents the climacteric

and ripening (30). During the pre-climacteric period the cells are not fully utilizing their work capacity as evidenced by the increased rate of oxygen uptake by whole fruit, slices, and isolated mitochondria after treatment with dinitrophenol. There is convincing evidence that at least part of the climacteric rise in respiration is caused by a decreased ATP/ADP ratio resulting from increased synthesis utilizing ATP. Prominent examples among these syntheses are the increases in aldolase and carboxylase in bananas observed by Tager & Biale (38), the increase in malate decarboxylase in apples observed by Neal & Hulme (39), and the synthesis of lycopene in tomatoes. Hulme (33) has pointed out that an increased rate of protein turnover would require energy at an increased rate and thereby promote an increased oxygen uptake without any increase in total protein. In considering protein turnover the recent observations that energy is required for protein degradation (40, 41, 42) become of unusual interest. The synthesis of highly reduced compounds, such as lycopene, from acetate (43) obviously requires energy, as does the synthesis of ethylene (44, 45), and these syntheses also must contribute slightly to a decreased ATP/ADP ratio. Until someone devises a means of measuring the work capacity of cells, one cannot rule out the possibility that a partial uncoupling of oxidative phosphorylation makes a contribution to the decreased ATP/ADP ratio and the increased oxygen uptake during the climacteric rise.

It is equally impossible to state the degree of coupling in the post-climacteric period although the electron transport function of the mitochondria is operating at full capacity, as indicated by the lack of effect of dinitrophenol on oxygen uptake of whole fruit, slices, and isolated mitochondria. However, it is clear that the post-climacteric mitochondria *in vivo* are not completely uncoupled in spite of the appearance of uncoupling agents in the cytoplasm. There can be little doubt that the cellular respiratory capacity and work capacity decrease during senescence, and it seems likely that this is caused by gradual loss of mitochondrial function with the loss of phosphorylative activity preceding the loss of electron transport capacity.

Further development of this approach must center upon the details of mitochondrial metabolism and its control. At this point it seems appropriate to anticipate the expansion of the discussion to include other senescent tissues—particularly leaves, flowers, and the cotyledons and endosperms of germinating seeds. The changes accompanying the gradual respiratory decline and senescence of these tissues, either attached or detached, are comparable to those that occur in ripening and postharvest fruit. In addition, in many instances, such tissues are easier to obtain and therefore are more amenable to laboratory investigation. The recent thesis of Gindel (46) that an important function of many fruits is to provide the embryo of the germinatng seed a supply of nutrients, renders especially apt the comparison between fruits and cotyledons (and endosperms).

Ample evidence now exists that synthesis of specific proteins occurs in

the cotyledons (47, 48, 49) and endosperms (50, 51) of germinating seeds, even though there is a simultaneous decline in the total protein content of these structures. In pea cotyledons one of the proteins synthesized is a phosphatase (another is amylase) with a pH optimum of 7 and a high specificity for ATP and ADP (47). This enzyme is apparently synthesized by the ribosomes, accumulates in the soluble fraction of the cytoplasm, and is distinctly different from the ATPase activity associated with mitochondria (48). Clearly it is a candidate for an important role in controlling the cytoplasmic ATP/ADP ratio and thereby, respiration and the efficiency of utilization of the energy that is conserved as ATP during oxidative phosphorylation. The synthesis of this phosphatase depends upon the energy supplied by mitochondria. In turn, the functional integrity of the mitochondria depends upon a heat stable organic factor that moves into the pea cotyledons during the first 24 hr. of germination (52). In the absence of this factor (that is, when the axis tissue is dissected away from the dry seed and the cotyledons are incubated under germination conditions) the mitochondria become "leaky" and begin to lose phosphorylative efficiency within 24 hrs. and are completely uncoupled within 38 to 48 hr. (48). After 72 to 96 hr. the cotyledons no longer take up oxygen (52). As the oxygen uptake decreases there is a concomitant rise in R.Q. The factor from the axis tissue has not been identified. Imbibition by the dry cotyledons of solutions containing 10^{-4} to 10^{-10} M kinetin, gibberellic acid, indoleacetic acid, napthaleneacetic acid, ascorbic acid, glutathione, and many other substances, was without effect in simulating the natural agent except in the case of 5×10^{-6} M napthaleneacetic acid (52). In this instance the respiratory decline and the increase in R.Q. are delayed until the sixth or seventh day. Cotyledons from which the axis tissue has been dissected after 18 to 48 hr. of normal germination will remain alive and healthy for as long as 4 to 6 weeks. The axis-free cotyledons could also be maintained in a healthy state (as indicated by their oxygen uptake, R.Q., ability to incorporate labeled glycine into protein, and the fact that active mitochondria could be obtained from them) for at least seven days by allowing them to imbibe $CdCl_2$ (10^{-2} M) or *p*-fluorophenylalanine (1mg/ml) during the first few hours of incubation. Although all of the present evidence (53) is against the possibility of the factor being an antifungal or antibacterial agent such as that reported by Spencer *et al.* (54), it is necessary to bear in mind the possible effects of the presence of such agents in mature and senescent cells. A soluble, heat-labile, nondialyzable substance that produces swelling and uncoupling of isolated mitochondria is found in the cotyledons of germinating peas after 10 to 18 days and in the axis-free cotyledons after 38 to 72 hr. (53). This substance is absent from the axis-free cotyledons pretreated with $CdCl_2$ or *p*-fluorophenylalanine. It does not appear to be an RNAase; extraction with iso-octane does not affect its activity, nor is the iso-octane extract active; it is strongly adsorbed by mitochondria; and its action on isolated mitochondria is prevented when it is preincubated with

10^{-4} *M* $CdCl_2$. To resolve the physiological meaning of these results one needs to know: (*a*) the source of the uncoupling substance—is it produced by mitochondria, microsomes, or nuclei—is it the cause or the result of *in vivo* mitochondrial inactivation? (*b*) what part of the mitochondrial structure is attacked by the substance, and (*c*) what is its relation, if any, to mitochrome (55, 56)? Through teleological spectacles it appears that pea cotyledons are supposed to die gracefully, gradually relinquishing their demand on their own reserves. Mobilization of these reserves requires the synthesis of new enzymes and at the same time, to insure the selfless participation of the cotyledon cells in the establishment of the growing parts of the seedling, it is their heritage to produce their own slow poison.

In castor bean and other oil-bearing seeds, synthesis of enzymes of the glyoxalate cycle is necessary before the triglycerides can be converted to, and mobilized as, sucrose (57, 58, 59). Mitochondrial uncouplers or inhibitors or both have been reported in ripening avocadoes (60), in rose petals (61), and in animal cells (56, 62). Further insight into the role of such substances in the respiration of postharvest tissues should be greatly aided by the development of satisfactory methods for isolating mitochondria from such fruits as apples (63, 64, 65), tomatoes (66), and bananas (63).

In addition to the specific examples already mentioned of enzymes showing a net increase in activity, there are many independent and more general indications of an active protein metabolism in mature and senescent tissues [see the reviews by: Bonner (67), Folkes & Yemm (68), Mothes (69), Pirie (70), and Yemm (71)]. In mature leaves excision from the plant initiates a rapid decline in total protein content that is not stopped by supplying mineral salts, carbohydrates, or nitrogen compounds, but is slowed by the administration of kinetin (69, 72), benzimidazole (73), and 2, 4-D (74), and is stopped by the formation of adventitious roots. During this period of decline the leaves retain a reduced capacity to incorporate N^{15}-labeled ammonia (75, 76) and C^{14}-labeled amino acids (77, 78, 79) into protein, and virus synthesis still occurs. Vickery (80) has shown that after the protein of detached leaves falls below certain levels the leaves are no longer able to recover from various stresses, presumably because of the irreversible loss of some necessary enzyme. Mothes' recent experiments (69) dramatically demonstrate the effectiveness of kinetin and benzimidazole in maintaining the capacity of *Nicotiana* leaves to withstand stress. These observations that protein turnover occurs in mature cells are consistent with the turnover detected in resting cells of yeast (81) and of *E. coli* (82).

In starving excised barley leaves, the rate of respiration is limited by available substrates; there is a shift of respiratory substrates from carbohydrates to amino acids derived from protein breakdown; and a marked accumulation of glutamine and asparagine is apparent (71). Administering glucose to detached wheat leaves causes an increased CO_2 output, with part coming from the administered glucose and part from the endogenous sub-

strates (83). There is a general increase with maturity in the level of proteolytic enzymes of root cells (84) and an increase with age of dipeptidase activity in *Elodea* (85). Likewise, a steady increase of the proteolytic activity of the mycelia of *Penicillium griseofulvun* and other fungi occurs with age. During the first few hours of nitrogen starvation a several-fold increase is noted that persists until nitrogen is again supplied (86). This increase in activity probably represents *de novo* synthesis and is not accompanied by net protein breakdown.

Changes in Specific Sub-Cellular Entities During Senescence

Mitochondria.—A central question in the study of senescence is the problem of the biogenesis and life span of mitochondria. Is there replacement of mitochondria in fully expanded non-dividing cells or are mitochondria produced only during cell division and cell enlargement? If the latter, what parts of the mitochondrion are subject to wear and repair and what parts to wear without repair? The present knowledge of the origin and ontogeny of mitochondria has been discussed (32, 67, 87, 88, 89) but as yet we have no satisfactory answer to the questions posed. It is clear that there is a great similarity in the structure and function of plant mitochondria derived from a wide variety of tissues, and that plant mitochondria are closely comparable to animal mitochondria (87, 90). There appears to be a definite development of the internal structure of mitochondria in the meristematic cells of both corn roots (91) and pea roots (92) from a more or less homogenous interior to one with well defined cristae. In the mature cells the mitochondrial interior again tends to be disorganized and a loss of respiratory efficiency (91) is evident. Howard & Yamaguchi (93, 94) have reported a striking increase in the oxidative activity of isolated mitochondria during the maturation of pepper fruit. The mitochondria from immature fruit, compared to those from mature fruit, contained less succinic dehydrogenase. Isolated plant mitochondria exhibit a characteristic osmotic behavior together with a change of dimension, structure, and biochemical capacity in hypotonic solution (31, 32), and isolated mitochondria are capable of an energy-dependent salt uptake (50, 95). The physiological significance of these properties is unknown. Isolated plant mitochondria incorporate labeled amino acids into mitochondrial protein (96, 97), and synthesis of some specific protein may be expected to occur because of the demonstrated net synthesis of cytochrome-*c* in isolated calf-heart mitochondria (98). Pertinent to the problem of the cause and nature of the changes in the mitochondria of senescent cells are the studies of Hanson (99) on the effect of ribonuclease on isolated mitochondria. Hanson found that corn root mitochondria contain about 1 per cent RNA and concluded that at least a part of this RNA is functional because of the deleterious effects of treatment with RNAase. Low concentrations of RNAase caused a slight rise in oxygen uptake and a decreased P/O ratio while higher concentrations (1 mg/ml) uncoupled phosphorylation and decreased oxygen uptake.

These effects were not attributable to the action of RNAase products, to adsorption of RNAase on mitochondria, nor to proteolytic contaminants of RNAase. In a later paper (100) Hanson suggests, on the basis of the effects on root tissue of RNAase, ethylenediamine tetracetic acid, and potassium chloride, that RNA associated with various membranes is essential to respiration, ion accumulation, and solute retention. He proposes that calcium or magnesium, or both ions bound to RNA protect it against RNAase and that the *in vivo* function of mitochondria is governed by the $K^{+}/Mg^{++}+Ca^{++}$ ratio. Zinc ions have also been implicated in the RNAase-RNA-protein relationships of citrus leaves (101) and it should be noted that an enhancement of the nuclease activity of bean hypocotyls by indoleacetic acid and kinetin has been reported (102).

Cytoplasmic membrane and cell wall.—Sacher has recently proposed that the changes accompanying senescence of fruits and abscission of leaves and fruits are due to a loss of integrity by the membranes, resulting from insufficient levels of auxin (103). This proposal is based on experiments with auxin-treated and untreated segments of the pods of Kentucky Wonder pole beans and leaf slices of *Mesembryanthemum sp.* and *Rhoeo discolor* (26, 103, 104). The untreated segments of bean endocarps became soft and flaccid and water-logged in the intercellular spaces within three days, while the treated (4 to 50 p.p.m. NAA or IAA) segments remained rigid and plump up to 17 days. A surface exudate appeared on the untreated segments, cellular dissociation occurred, and the leakage of solutes was three times that from the treated segments. Furthermore, endogenous respiration ceased much earlier in the untreated samples. Similar effects of IAA and NAA in preventing degradative changes were obtained with *Mesembryanthemum* and *Rhoeo.* These experiments do not foster the conclusion that the cytoplasmic membrane is the only membrane affected because many of the same effects might be expected if either the tonoplast or the mitochondrial membranes were impaired. Nor do the results necessarily indicate that loss of membrane permeability is the primary event in the degradative changes that occur in the absence of added auxin; however, the effects of IAA and NAA in delaying senescence are clearly demonstrated.

Osborne has shown that senescence in both attached and detached leaves of several deciduous species may be delayed by application of 2,4-D or 2,4,5-T (74, 105). The treated areas of the leaves remain green, retain both a relatively high rate of oxygen uptake and the ability to fix carbon dioxide photosynthetically, while the surrounding untreated tissue undergoes normal senescence. The aging processes have been arrested in detached leaves of *Xanthium* by treatment with kinetin (72) and in detached wheat leaves by treatment with benzimidazole (73). Leaf yellowing and abscission have been delayed in several species by spraying with gibberellic acid (106), and the delay of the onset of senescence by removal of the flowers has recently been re-investigated (19, 107). These experiments indicate that senescence, as

well as growth and differentiation, is linked to the hormone level of the tissues but they do not yet elucidate the series of events at the molecular level.

The natural fall in the gradient of pectin methylesterase activity that occurs across the abscission zones of petioles of *Phaseolus vulgaris* can be prevented by treatment with 2,4-D or 2,4,5-T and abscission is prevented (108). Yager (109) has proposed the existence of other enzymes active in abscission. Pectin methylesterase is present in the exudate of water-logged (low auxin) bean endocarp segments (104). These observations suggest that a low level of auxin may bring about a change in the distribution and activity of pectin methylesterase (and possibly other enzymes) and thereby cause a change in pectin metabolism that would contribute toward the changes occurring in senescence and abscission. Glasziou (110) has reported that at least 90 per cent of the pectin methylesterase of intact oat coleoptiles is bound, probably to the cell wall, and that the degree of binding is dependent on the level of IAA. Jansen *et al.* (111), however, found no influence of IAA on the binding of pectin methylesterase by *Avena* coleoptile cell walls. Whatever the relationships between senescence, auxin, and pectin methylesterase, it is now becoming evident that several enzymes are localized primarily or exclusively in the cell wall. Invertase appears to be located at the surface of cells of *Canna* leaves (112) and in the free space of wheat roots and coleoptiles (113), while an extracellular amylase has been reported in cultures of bean stem segments (114). Pyrophosphatase, ATPase, and uridine diphosphoglucose pyrophosphorylase activities are associated with corn coleoptile cell wall fragments [Kivilaan, Beeman & Bandurski, quoted in (110)], and ascorbic acid oxidase and hexose phosphatase activities with the wall fraction of sycamore cells in solution culture (115). It is interesting to note that the hydroxyproline of plant cells is also located in the cell wall proteins (116, 117). Therefore we have an increasing scope for ideas on the possible action of auxin in controlling growth and senescence through cell wall metabolism.

Nuclei, endoplasmic reticulum, and ribosomes.—It is becoming increasingly evident that the various structures of the cell are intimately and subtly interrelated, both structurally and functionally (87, 89, 118, 119). There is strong evidence that a part of the vesicular network of the endoplasmic reticulum is continuous with the cytoplasmic membrane and extends into and through the walls and that this portion of the reticulum may constitute the limiting membrane between the apparent free space and the remainder of the cell volume. Another portion of this vesicular network of membranes is apparently continuous with the nuclear membrane. The ribonucleoprotein particles of the microsomal fraction appear to be produced in the nucleus and expelled into the cytoplasm through the nuclear pores (14, 120, 121). Within the nucleus the nucleoli are the centers of the most intense RNA metabolism (14) while protein synthesis occurs in particles similar to the ribonucleoprotein particles of the microsomal fraction (122, 123). To what extent the cytoplasm of somatic cells is dependent upon

a continuous supply of RNA from the nucleus remains to be established. A ready pathway probably exists for the exchange of coenzyme and substrate molecules between the nucleus and the cytoplasm. In this closely interrelated continuum of structural elements and enzymatic functions it is not surprising that many treatments, e.g., removal of the nucleus, addition of metabolic inhibitors, and starvation, bring about a metabolic decline similar to that of normal senesence, and that a decrease in the levels of many enzymes and cofactors can be correlated with senescence.

Because the bulk of the cellular protein is synthesized by the ribonucleoprotein particles it is of interest to study this synthetic capacity in senescent cells. The microsomal fraction, isolated from post-climacteric avocado fruit in which the mitochondria have lost the ability to carry out oxidative phosphorylation *in vivo* and *in vitro*, is still capable of an ATP-dependent incorporation of labeled amino acids into protein (124). Exactly similar but more detailed results have been obtained with the microsomal fraction from pea cotyledons in normal and induced senescence (48, 53). Within the limits of such a test to indicate the functional integrity of ribonucleoprotein particles, one can conclude that the particles are still capable of functioning *in vivo* but cannot because of the lack of ATP. Such a conclusion is consistent with the known stability of isolated ribonucleoprotein particles (125), their capacity for dissociation into subunits, and their reassociation to form functional particles (126). A conclusion of that nature also draws support from the report of only a slight decline in both RNA content and the amino acid activating enzymes of rat liver during extensive protein deprivation of the rat (127). Further evidence that the microsomal fraction is rather stable derives from the observations that anucleate fractions of *Acetabularia* live for several months and during this time bring about a net synthesis of several enzymes (14). Intriguing as these experiments are, they do not begin to deal with the possibility that several (or many) kinds of ribonucleoprotein particles with different stabilities exist in the same cell or that there are different kinds in different cells of the same plant (128).

However, although we have substantial evidence for the role of the nucleus in controlling metabolic events in the extra-nuclear portion of a cell during mitosis, growth, and maturation, there is virtually no information on the functional state of the nucleus in a senescent cell. Huang (53) has found no change during senescence in the ability of the nuclei of pea cotyledon cells, either *in vivo* or *in vitro*, to incorporate tritiated thymidine. Again, within the limits of the test to indicate the functional integrity of the nucleus, the results indicate that loss of nuclear activity is not the primary event in the senescence of pea cotyledons. It is perhaps of interest to recall that the senescence of pea cotyledons can be delayed by napthaleneacetic acid, by a factor from the axis tissue (48, 52), and that Thimann and Beth have shown that anucleate fragments of *Acetabularia* still respond to treatment with indoleacetic acid (129). This section can be concluded by noting that these

studies will now be greatly facilitated by the availability of adequate methods for the isolation of nuclei (130) and nucleoli (131) from plant tissues.

Chloroplasts.—Because senescence is not limited to green cells, chloroplasts obviously are not requisite structures for this phenomenon and they deserve attention from the standpoint of their possible capacity for modifying senescence. To the well established observations that chloroplasts are self-propagating entities (120) capable of photosynthetic phosphorylation and photosynthetic carbon dioxide fixation (132), we must now add the recent observations that chloroplasts contain amino acid activating enzymes (133) and conduct a light-dependent incorporation of amino acids into protein (97). They may therefore synthesize much of their own protein, RNA, and perhaps DNA as well (134). Although only a small fraction of the total RNA of a green cell is localized in the chloroplasts a close correlation is evident between the total RNA of a green leaf and its capacity for photosynthetic phosphorylation (135). Hence the RNA content of a green leaf appears to be a measure of its photosynthetic ability as well as an indication of its capacity for respiration, oxidative phosphorylation, protein synthesis, and cell division. In drought and senescence a more rapid loss of protein takes place from chloroplasts than from the remainder of the cell (136). In detached leaves, however, there is a parallel in the rate of protein loss in chloroplasts and cytoplasm (136). Relevant to the earlier discussion about protein catabolism requiring energy, Wood & Cruickshank (136) have observed that detached leaves in nitrogen showed no loss of protein although there was a decrease in carbohydrates. It will be interesting to learn whether chloroplasts contain an energy dependent system for protein catabolism. Apparently we have too little information to allow a decision on the possibility that chloroplasts and mitochondria can serve as alternate and independent sources of ATP for the work requirements of a cell, although Smillie (135) concludes that it is unlikely.

Vacuoles.—The vacuoles probably cannot be considered functional entities in the sense that they carry enzymatic apparatus necessary for the synthesis of cell stuff, yet it is clear that the integrity of the tonoplast must be maintained to prevent the release of a flood of noxious substances into the cytoplasm. The osmotic properties of vacuoles have been clearly demonstrated (87). The origin of the tonoplast and the means of the maintenance of its permeability properties are highly uncertain (87, 90). Probably many events observed in the final stages of senescence result from the leakage of such substances as phenols from the vacuole and their consequent interaction with the remaining functional elements of the cell (137, 138). It seems likely that this leakage is secondary to the loss of energy necessary to maintain the membranes. Publication of a method for obtaining isolated vacuoles (87, 139) should permit a more detailed study of their properties.

Cytoplasmic ground substance.—This fraction is poorly characterised at present and includes the left-overs after clearly recognizable structures have

been accounted for. Current indications are that this fraction too possesses a structure not quite resolved by present methods (87, 90, 140). This matrix separates and supports the particulate structures of the cell, contains many of the "soluble" enzymes, and absorbs and perhaps produces the shears and stresses accompanying cytoplasmic streaming (140). Loss of activity by one or more of the enzymes belonging to this fraction might be sufficient to trigger the onset of senescence and death. It appears, though, that this portion of the cell is not self-reproducing and the enzymes present are synthesized by other cellular structures. In this view, changes observed in the ground substance, even though correlated with senescence, would be derivative rather than primary.

Changes in Metabolic Pathways

Studies of the effects of various metabolic inhibitors on young and old plant and animal tissues suggest a gradual shifting of metabolic pathways during aging (27). The data of Tager & Biale (38) indicate a shift from the pentose phosphate pathway to the Embden-Meyerhof-Parnas (EMP) pathway in the ripening banana; carboxylase and aldolase activities are very low in the immature banana but increase several-fold during and after the climacteric rise in respiration. By measuring the relative rates of production of labeled carbon dioxide from glucose-6-C^{14} and glucose-1-C^{14} it is possible to determine the relative contributions of the pentose phosphate pathway and the EMP pathway in the degradation of glucose (141, 142). Embryonic plant tissues, especially roots, seem to degrade glucose exclusively by the EMP pathway with an increasing importance of the pentose phosphate pathway as germination proceeds (143). Gibbs & Earl (144) have reported changes of sufficient magnitude in the activities of aldolase, 6-phosphoglucose dehydrogenase, ribose-5-phosphatase, and fructose-1,6-diphosphatase of developing pea roots to provide a convincing explanation for the change in pathways. Alternatively, the controlling factor in the proportioning of the contribution of the two pathways might be the $TPNH/TPN^+$ and $DPNH/DPN^+$ ratios (144). In the experiments of Gibbs & Beevers (143) added indoleacetic acid had no influence on the pathway of glucose metabolism. On the other hand, such an influence has been observed by Humphreys & Dugger (145) and by Shaw *et al.* (146). The work of Wang and his associates demonstrates the presence of the pentose cycle pathway in mature pepper and tomato fruit (147, 148). The importance of the synthesis of the enzymes of the glyoxalate cycle in the conversion of fatty acids to carbohydrates in the endosperms of oil-bearing seeds (57) has already been mentioned. In the petals of cut roses a decrease occurs with age in the malate to citrate conversion, as well as in the dark fixation of $C^{14}O_2$ (149). The increased malate decarboxylase in apple tissue (39) apparently diverts malate into a decarboxylation pathway that results in an increased R.Q. for the tissue.

Recent reviews have dealt at length with the various possible pathways

for the transport to oxygen of hydrogen or electrons from reduced cofactors in a variety of plant tissues including senescent tissues (31, 32). The fact that young and old tissues differ in their responses to various respiratory inhibitors (27) suggests at least a change in the rate-limiting reactions, if not a shift in electron transport pathway. These changes are almost certainly of great significance in the metabolism of plant tissue and Hackett's excellent review (32) should be consulted for a complete discussion of the problem.

It is perhaps useful to be reminded that changes in the intensity of various metabolic pathways can occur without changes in the concentrations of the enzymes involved (150).

Changes in chemical composition.—Ulrich's review (34) considered the changes in chemical composition of ripening fruit in detail, and Kefford (151) has reviewed the work on the chemical and enzymatic constituents of citrus fruits. Since these reviews there has been a painstaking account of the histological and histochemical changes in developing and ripening peaches (152, 153, 154), further delineation of the changes during growth and storage of avocado fruit (155), a detailed analysis of the chemical changes in cut roses (156) and aging maple leaves (157), as well as a heroic documentation of the chemical composition of the leaves (158) and fruit (158, 159) of the banana, and of the influence of various factors on their composition (160, 161).

Biogenesis of Ethylene

Ethylene is a normal metabolic product of plant tissues (162, 163, 164) and the gross effects (epinasty, acceleration of ripening) of its physiological activity are well known (30). Whether the quantities of ethylene present in plant parts other than the fruit are of any physiological significance under usual growing conditions is still an open question. Many kinds of ripening fruit, however, accumulate sufficient ethylene to influence the rate of ripening (162). It is uncertain whether ethylene initiates this ripening or is simply a by-product of it. Because of the physiological activity of ethylene and because it is produced in fruit only during ripening, both its biogenesis and its further metabolism are of great interest.

The development of micromethods for the separation, identification, and estimation of ethylene (66, 165) has resulted in significant progress in the problem of ethylene biogenesis. Using a gas chromatographic procedure (165), Burg & Thimann (44, 166) studied the conditions affecting ethylene production by plugs of apple tissue. The process was strongly inhibited by low oxygen tensions and ceased entirely under completely anaerobic conditions. Storage for 1 to 4 hr. in nitrogen and subsequent return to air brought about an increased rate of ethylene production, suggesting that an ethylene precursor accumulates under anaerobiosis. After a 4-hr. exposure to nitrogen, a 5-min. exposure to air allowed ethylene production to continue for about 60 min. Soaking the plugs in water for one hour greatly decreased ethylene production, while soaking in glycerol, potassium chloride, and a variety of

other substances prevented such a decline. Because the decline brought about by soaking in water could be reversed by a subsequent soaking in glycerol, the decline did not result from a loss to solution of ethylene precursors. Experiments with a range of concentrations of glycerol and potassium chloride indicated that a certain minimum tonicity of the cell sap must be preserved in order to maintain ethylene production. Higher tonicities were not inhibitory. The threshold concentration for the inhibition of ethylene production and oxygen uptake was the same for six different metabolic inhibitors and ethylene production and respiration were closely parallel over a large concentration range of each of the inhibitors. Also, the responses of the two processes to oxygen tension were nearly identical. These properties of the ethylene-producing system led Burg and Thimann to suggest that (*a*) it is located in a particle, (*b*) it is inactivated by swelling of the particulate structure, (*c*) it is closely linked with a terminal oxidase, and (*d*) it is dependent upon the respiratory process for energy. The observation that the inhibitions caused by dinitrophenol and fluoride were partially reversed by ATP (and other energy sources) further indicates that ethylene production requires respiratory energy. The experiments of Spencer (45), in which whole mature green tomatoes infiltrated with dinitrophenol showed an immediate depression of ethylene evolution, also indicate a requirement of oxidative phosphorylation for ethylene production.

While the observations of Burg and Thimann are strongly indicative of the site of ethylene synthesis, the data of Spencer (66) seem to establish the mitochondrion as this site. She reported that mitochondria isolated from tomato fruit produce ethylene in quantities equivalent to those produced by the whole fruit and the pattern of ethylene production by mitochondria isolated from tomatoes of different physiological ages closely parallels the evolution of ethylene from whole fruit of comparable ages. However, Meigh *et al.* (167) observed no ethylene production by isolated apple and tomato mitochondria even though the mitochondrial preparations were capable of oxidative phosphorylation and the crude homogenates from which the mitochondria were prepared produced easily detectable quantities of ethylene.

The small quantities of ethylene produced in plant tissues make the study of ethylene metabolism difficult even with radioactive tracers. A few such studies have been reported, however, and indicate that the immediate precursors of ethylene are not closely related to any known major metabolic pathway. No radioactivity could be found in ethylene produced by plugs of apple tissue after treatment with 1,3-C^{14} glycerol and 2-C^{14} glycerol although the label was found in glutamate, aspartate, malate, fructose, glucose, sucrose, alanine, and succinate (168). When labeled sucrose and acetate were used in long term experiments (22 to 58 hr.) the ethylene produced was labeled. The ratio of the specific activity of ethylene to that of carbon dioxide was much higher for the labeled sucrose (0.25–0.29) than for the labeled acetate (0.10–0.0025) experiments. Labeled tyrosine did not give rise to labeled

ethylene. These results indicate that the direct precursors of ethylene must be sought among compounds other than the intermediates of glycolysis, of Kreb's cycle, and of the degradation of aromatic compounds. In apple tissue the tritium of tritiated water is rapidly incorporated into ethylene and reaches complete equilibration in about three minutes (166). Consideration of both the physiological properties of their system and the data from the carbon and tritium tracer experiments led Burg and Thimann to propose the following sequence of steps in the biosynthesis of ethylene (166).

$$A \xrightarrow[-2H]{} B \underset{+H_2O}{\overset{-H_2O}{\rightleftharpoons}} C \longrightarrow \text{ethylene}$$

The evidence suggests that: compound A is normally present in low concentration but accumulates under nitrogen; the conversion of A to B requires oxygen and is relatively fast; the rate-limiting step under normal conditions is the formation of A; after prolonged exposure to nitrogen, the conversion of B to ethylene is rate-limiting; and one of the final steps is a reversible dehydration. The rapid equilibration of the ethylene with tritiated water might be the result of the binding of ethylene to an enzyme in such a way as to enhance the already acidic nature of the ethylene hydrogen.

It is intriguing to speculate further about some of the possible pathways for ethylene biogenesis. The oxygen requirement and energy requirement of the ethylene producing system and the tracer experiments seem to rule out the synthesis of ethylene by the simple dehydration of ethanol. The elimination of ammonia from ethylamine by an aspartase-like reaction seems equally improbable. The recent demonstration of the formation of carbon-carbon double bonds by a non-hydrolytic elimination of phosphate (169, 170) raises the possibility that 3-phosphopropionic acid might be an intermediate in analogy with the formation of isopentenylpyrophosphate (170). Acrylyl CoA formed in the conversion of β-alanine to propionic acid (171, 172) might also be considered.

$$\beta\text{-alanine} \rightarrow \beta\text{-alanyl-CoA} \rightarrow \text{acrylyl-CoA} \rightarrow \text{acrylic acid} \rightarrow \text{ethylene}$$

Whatever the pathway for the biogenesis of ethylene, the enzymatic apparatus involved is presumably highly specific since there is only a scant production of other olefins. Huelin & Kennet (173) report that if C_3 to C_6 olefins occur in apples the proportion must be less than 0.1 per cent of the ethylene, while Meigh (174) found acetylene, ethylene, propylene, and propane in the quantities of 20, 27,000, 9, and 7 mμg/kg/hr produced by apples in air at 20°C. Meigh (174) also reported that potatoes produce a variety of hydrocarbons.

Not only is the rate of production of ethylene slow and the biosynthetic pathway obscure, but the further metabolism is slow and the pathway unknown. After exposing avocadoes, pears, oranges, limes, papayas, apples, tomatoes, and grapes to one part per thousand ethylene-C^{14} (1.4×10^8 counts

per minute per millimole) for several days, only avocadoes and pears showed any measurable incorporation of radioactivity and this was mainly in the organic acid fraction (175). Degradation of the succinic and fumaric acids from the avocado fruits showed 77 per cent of the label in the carboxyl groups in both cases. The respired carbon dioxide was inactive in all experiments. Buhler *et al.* (175) concluded that ethylene is probably an end product of plant metabolism and the small incorporation that is observed results from exchange reactions in the biosynthetic sequence of ethylene. Meager as these results are, they do demonstrate that experiments with labeled ethylene are feasible. Further work along these lines, using ethylene of the highest possible specific activity so that the kinetics of the uptake and distribution of the label might be followed, should be rewarding.

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PLANT WATER DEFICITS AND PHYSIOLOGICAL PROCESSES[1,2]

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The plant component of the soil-plant-atmosphere system is an organized body or structure permeated by water. When we speak of a plant as an organization we refer to evidence of the integrated unity that is apparent throughout its entire phenologic cycle (186).

In the view of Needham (112) the plant (as a micro-universe) can be considered as having a number of levels of organization and complexity from the atomic to the whole organism. Laws applying to one level may not apply to other levels. Each level requires its own concepts. Any study of more than one level at a time must involve an attempt to determine the intrinsic relationships between one level of complexity and the others, and, finally, the organizations perceived at each level of complexity must be integrated.

These same considerations should govern our concepts of the relation between the plant and the soil and atmospheric environmental factors affecting its life, with water recognized as a vital factor that is both intrinsic and extrinsic. Water is essential to the life and growth of plants, being a major constituent of living green tissues and often comprising as much as 90 to 95 per cent of the fresh weight. Water provides plants with their mechanical strength through the mechanism of cell turgor, since most vegetative tissues are composed of cells with non-lignified walls. Such plastic bodies become mechanically stable under the influence of the hydrostatic pressure developed by water inside the cell. Water is a raw material in metabolism and in the synthesis reactions that occur in living plants, even though the amounts involved here are rather small compared to the total amount of water that passes through the plant. Large amounts of water move into the roots, through the stem, into the leaves, and out into the atmosphere in the form of vapor, in what is known as the transpiration stream. Growth itself is an expression of cell enlargement brought about by the action of water. Many of the metabolic reactions of plants are conditioned by the degree of hydration of certain of the reactants or enzymes (23, 185).

To understand this complex system we must approach it with the realization that it is highly dynamic and composed of many interlocking and interdependent phenomena. One of the most difficult problems encountered in understanding the behavior of water in the soil-plant system arises because observed reactions involving water have to be integrated both over time and through volume (141).

[1] The survey of literature pertaining to this review was completed in September 1960.

[2] The following abbreviations are used: DPD (diffusion pressure deficit); OP (osmotic pressure).

DEVELOPMENT OF INTERNAL WATER DEFICITS

The Water Pathway

The plant transpiration stream amounts to an aqueous leak, an aqueduct, between the soil moisture "reservoir" and the atmospheric "sink." Liquid water moves from the soil interstices to the root surface, through the root epidermis and cortex, and into the xylem elements of the stele. The water then ascends through the xylem strands to the leaf, crosses the cells of the mesophyll tissue, and moves through the walls of the cells bordering the substomatal chamber (35, 36). At this point water sustains a relatively large loss of free energy, changes state from liquid to gas, and by random thermal movement diffuses through the gas-filled substomatal chamber, passes the stomatal orifice, or follows constricted pathways through the epidermal cuticle into the free atmosphere. This is the generalized, net pathway of water down its gradient of free energy from one end to the other of the soil-plant-atmosphere water-conducting system.

The process of water movement through the soil-plant-atmosphere system is primarily energy-dependent. The energy to drive the system comes from the sun. Nevertheless, although the process is energy-dependent, it is commonly rate-controlled. The transmission characteristics and the potential gradients that can develop in the various parts often become the controlling factors in the movement of water through this system (141).

Transpiration appears to be unavoidable. Plant functions require an exchange of gaseous carbon dioxide and oxygen. This gaseous exchange is largely limited to stomata, and the largest portion of the surface of most land plants is such that water loss tends to be reduced to a minimum. Transpiration occurs simply because plants have evolved no method of exchanging certain gases without losing water vapor (194).

The water content at different loci in the plant is a phenomenon traceable all the way back to the absorption gradient found in the integument of the seed. From its youngest stages a seedling exhibits a structural polarity, the radicle constituting an absorbent pole and the epicotyl, the transpiratory pole. Between them exist a basipetal absorption gradient and acropetal transpiration gradient (133).

Water flow in plants is a process made more complex by interchanges of water between the various tissues all along the flow path, by changes in solute content caused by metabolism, and by temperature gradients throughout the plant. The flow phenomena are altered by changes induced by the water economy of the tissues or organs themselves. Such "feedback" control is typified by the reaction of the stomata to changes in the internal water stress of the leaf tissues. Furthermore, if the rate of water loss exceeds the rate of water supply, changes in the pattern of water movement occur because of shifts in the osmotic concentration of the cells or because of variations in the transmission characteristics of the tissues. Such changes usually restore the water balance of the tissues affected or of the plant as a whole.

This kind of continuous readjustment is one reason why the plant water balance is such a complicated system to describe quantitatively (141).

Relative Rates of Transpiration and Absorption

As the plant transpires, the course of the water loss process depends on the balance between water absorption, the rate of movement in the plant, and the evaporation from exposed surfaces. Under steady state conditions these processes proceed at the same rate, and both absolute water content and the relative distribution of water in the plant remain constant (87).

Absorption of water by plants is initiated and controlled primarily by transpiration. This is usually considered a result of evaporation of water from the mesophyll cells in the leaves, tending to cause a decrease in cell volume, a decrease in turgor pressure, and thus a fall in cell water potential with a general lowering in the value of the leaf water potential (increased DPD). The potential gradient thus established from the mesophyll cells to the leaf xylem traces is implicitly transmitted through the intact water columns of the plant xylem across the root cortex to the soil water. Water then moves in an integrated manner from the water reservoir in the soil surrounding the roots along this water potential gradient through the plant and into the atmosphere.

A key point in this construct is the implicit assumption that the water columns are intact from the mesophyll cell to the soil water. Greenridge (50) and Loomis (96) recently reviewed the various controversies regarding the cohesion concept. Whatever the doubts concerning the exact mechanism of water movement, there is no doubt of the close relationship between absorption and transpiration.

Many studies have properly emphasized that anatomical features of the plant and external environmental factors are important in transpirational water loss, and therefore also affect root water absorption, but the lag of absorption behind transpiration is determined by the sum of the resistances along the path of water movement. The rate of water movement to the root surface and the rate of root growth into moist soils will control this lag (130, 131). When the root surfaces are abundantly supplied with water at high potential the major resistance to water uptake is in the root since killing or removing the roots reduces the absorption lag (83, 86). Reduction of water absorption by metabolic inhibitors (86) and the increase of water absorption following the death of the roots indicates that in some way the root resistance to water absorption is associated with living cells of the root.

Although a supply of growth materials, maintenance of a high degree of hydration, proper rate of oxygen supply, a suitable temperature range, and limited mechanical impedance are important for root extension, and thus for water uptake in general (23), root elongation decreases as the water potential of the rooting medium decreases (47). When cell turgor pressure approaches zero, root elongation ceases (151). Subsequently the intrinsically slow rate of moisture flow in unsaturated soil will govern the rate of water absorption by

the root, irrespective of transpirational pull on the plant water columns.

Internal competition for water.—Whenever a water deficit develops in plant tissue as net leaf water loss exceeds absorption by the roots, differential gradients of vapor pressure develop between various cells, tissues, and organs of the plants as a result of an increase in cell sap concentration, decreased wall pressure, and imbibitional forces in the cell wall materials. These changes in gradients of vapor pressure incurred with developing water deficits are simply augmentations of differential gradients that have existed since the embryonic state of the plant and are continuously shifting in the dynamic plant system in response to changes in water movement rates, transpiration rate, concentration of cell sap, and turgor fluctuations (133).

When internal water deficits develop, whatever the cause, the water entering the plant root flows to points down at the end of the steepest water potential gradient. Internal plant body-water redistributes in the same way. Young leaves, growing tips, and young fruits (5, 10, 95, 139) usually exercise priority, and older leaves, shaded leaves, and old fruits suffer increasing internal water deficits until at some point they die and drop (73). A pattern of diurnal reversal in dehydration may occur, but the relative ranking of organs as to priority for water absorbed remains (192).

While the net effect of water deficits in plants is known in some detail, little exact information is available on local deficits, potential gradients, rates of water movement, water biological residence times, and variabilities in water turnover rates in plants under stress.

Cyclic patterns of water loss.—Dehydration of plant tissue can occur because of (*a*) maturation and aging, (*b*) seasonal deficits, (*c*) diurnal variations in water content, and (*d*) sudden deficits for short periods when the vapor pressure gradients from leaf atmosphere are suddenly increased before stomatal closure can compensate for the sudden increase in water loss. Meyer (106) recently reviewed this subject in detail. Only a few comments are made here.

Young tissues are relatively highly hydrated, with thin cell walls and the reduction in water content (on a dry matter basis) as maturation proceeds may be very great because of the accumulation of cellulose. Although these changes are probably not entirely because of ontogeny, the relation between ontogeny and water content deserves more attention than it has received (87).

In dryland plants, seasonal water deficits can develop that are not entirely ontogenetic but are largely phenologic expressions of a progressive seasonal gap between transpiration and absorption. This phenomenon has been studied largely in trees (45), in which water content on a dry matter basis reaches a minimum in the fall and a maximum in the spring (37, 140).

Well marked diurnal variations in the water content of leaves, stems, and roots are found in transpiring plants of all types, with maximum water content during the night and maximum deficit during the photoperiod (193). It is still puzzling that leaf turgor is maximum near midnight, with a decrease

toward morning (160, 193). A redistribution of water within the plant may be the cause.

In addition to ontogenetic, seasonal, and diurnal variations in plant water content, certain rhythms in water content (147), turgor (33), transpiration (108), cyclosis (180), exudation (4, 53, 54, 173), salt content of exudate (173), and permeability (161) have been observed. They seem to be connected with rhythms in metabolic activities.

Another source of change in plant water content can be sudden increases in the vapor pressure gradient from the leaf to the atmosphere when micro-air mass characteristics are suddenly altered by shifts in wind direction that bring more energy and drier air and sweep away the moist air blanket about the leaves. This effect merits study, particularly in regions where the recurrent damage from dry winds (55) might be reduced by selection of plant varieties, shifts in planting varieties, shifts in planting date, or water management during the most dangerous period in terms of plant development.

Factors Affecting Water Flux

Rate of water loss.—The literature on the effect of climatic and edaphic factors on plant transpiration has been reviewed in detail (137, 156, 157, 163). Environmental factors such as temperature, vapor pressures, and wind speed affect water loss principally through the physical process of evaporation. That leaf water loss obeys Fick's law of diffusion has been demonstrated theoretically and to some extent experimentally (9).

Other factors such as light intensity and plant water supply also affect transpiration but largely through the physiological processes connected with adjustments of the stomatal apparatus and concomitant changes in stomatal diffusion resistances.

With an abundant water supply the plant stomatal mechanism is thought to be activated by a light-sensitive reaction (157) connected with photosynthesis (180). On the other hand, when the plant is subjected to a severe water deficit between transpiration and absorption, the regulator of stomatal aperture appears to be an osmotic system (88).

Rate of water absorption.—When the transpiration rate is low or when the soil moisture potential is high, the effect of soil moisture variations upon water deficits can be expected to be slight, since soil moisture is not the factor limiting transpiration. Under high transpiration conditions (high light intensity, high temperature, low external vapor pressure) the effect of soil moisture tension can be expected to be great (13). Kuiper & Bierhuizen (88) showed rather convincingly that the soil water supply affects the transpiration of tomato shoots independently of light intensity. This may be achieved either by a drying of the cells surrounding the intercellular spaces, a decrease in the vapor pressure of the mesophyll cell (incipient drying), or a decrease of stomatal aperture with a decrease in vapor conductivity (reciprocal resistance) from the leaf to the atmosphere.

Slatyer (154) has just reviewed the literature on the absorption of water

by plants and movements of water through and out of the leaves. Only a brief discussion is required here to preface later remarks on the metabolic effects of water deficits developed in plants.

Since the coupled processes of absorption and water loss are controlled by quite different sets of factors it is to be expected that they get out of phase. A lag of absorption behind transpiration loss on hot days can occur even when the plant is growing on moist soils or in a nutrient solution. Such deficits are usually eliminated by absorption at night. As soil moisture is depleted, absorption becomes slower and slower and the deficit persists later and later into the night until permanent wilting occurs. Plant deficits can be caused by excessive rates of water loss or slow absorption, or both. Internal deficits caused by excessive transpiration are usually shorter and less severe than those caused by deficient absorption, although periods of hot, dry, windy weather can cause severe damage (1, 55, 77). The effect of soil moisture supply can be greatly modified by the atmospheric conditions that affect the rate of transpiration (91). Therefore, plant behavior cannot be explained in terms of either soil or atmospheric conditions alone since the internal plant water balance is an integration of all factors affecting plant-water relations (57, 59).

Because of resistance in the plant to water movement, and a consequent lag of absorption behind transpiration, the water content of transpiring plants almost always decreases during the day and rises again at night, when the stomates are closed and absorption exceeds water loss. A controversy continues in the literature on the importance of dew or fog absorption as a source of water for leaf rehydration. Gessner (44) and Stone (165) recently reviewed the conflicting evidence on this subject. There seems to be some beneficial effect on plants, but it is uncertain to what extent this is due to reduction in transpiration by diversion of morning insolation into evaporation of dew and to what extent dew is involved in rehydration of leaf cells (153).

Leaf water content.—Studies of Milthorpe & Spencer (107) showed that wheat stomata open when the leaf is wilted rapidly, but not when the leaf wilts gradually. Stomatal movements of wheat appeared to be largely independent of the level of leaf water content in the range of 70 to 90 per cent. These workers argue that it is "the *rate* and direction of change in local leaf water content rather than the magnitude of the water deficit of the leaf as a whole that is important in stomatal movement and thus transpirational water loss." Sudden increases in the vapor pressure gradient as the driving potential for water loss at the leaf-air boundary are perhaps not thought of as common. In arid environments, however, wind direction may govern the degree of energy advection, and shifts in the atmospheric vapor pressure at the leaf surfaces may be sudden and repeated, and may even affect over-all plant hydration drastically. Such an effect would likely be most damaging to exotic mesophytes with thinner cuticles. More is said about the effects of leaf water content in the discussion of stomatic control of leaf water loss.

Water Transport as an Integrated Catenary Process

The concept of water transport along a soil-plant-atmosphere thermodynamic continuum is implicit in the viewpoints of Gradmann (49) and van den Honert (70), and later Philip (130). These investigators analogized water transport to Ohm's law by considering that the rate of flow of water through any part of the soil-plant-atmosphere system is proportional to the potential difference, and varies inversely as the resistance to flow. Van den Honert regarded water transport as a catenary process in which the locus of greatest resistance to movement is the focal point for control of water movement. Other resistances would be of secondary importance to flow. Even with stomates open and the soil as dry as the permanent wilting percentage, the resistance to water movement across the gaseous phase from the leaf cells to the atmosphere is by far the greatest resistance encountered in water movement from the soil through the plant to the atmosphere.

Slatyer (154) commented on van den Honert's construct in some detail. Slavik (155) and Bonner (14) argued that the major resistance to water loss is at the leaf surface. Experimental data do show that absorption and transport can be greatly reduced by low soil temperature, inadequate aeration, or low soil moisture potential (84), which probably operate indirectly to reduce leaf water loss by reducing leaf water content and inducing stomatal closure.

Van den Honert pointed out that the gaseous phase is the logical place for regulation of plant water loss since control exercised elsewhere would result in dehydration of the plant between the leaf surface and the regulatory focal point. The only other segments of the transport pathway where resistances to flow seem likely to be important are at the soil-root surface (131) and in the zone between the root surface and the xylem where resistance appears low whenever environmental factors are not adversely affecting metabolism. The most important over-all factor affecting water transport appears to be a low soil-water potential (high soil moisture stress) at the root surface. This potential affects (*a*) plant water potential (DPD) directly, (*b*) plant hydration, which in turn affects permeability and thus root resistance to water flow, and (*c*) rates of water transport through the soil to the root surface.

MECHANISMS FOR WATER LOSS CONTROL

Although we observe that water is taken up as a liquid at one end of the plant "aqueduct" and is discharged at the other end as vapor, plant physiologists also observed long ago that the plant exercises a degree of control over the loss of water at the leaf-atmosphere boundary. Commonly, leaf water loss was found to follow a cyclic, apparently exogenous rhythm correlated with the diurnal photocyclicity. An endogenous rhythm of water loss has also been identified (108), and the phenomenon is currently again under study in the authors' laboratory.

The leaf apparatus involved in water loss control consists of a few rather

simple anatomical components. Since the time of von Hoehnel (69, 162) we have distinguished between stomatal and cuticular transpiration, stomatal transpiration being determined by the dimensions of the stomatal orifice, and cuticular by the permeability of the waxy covering of the leaf surface. Leaf cuticle has long been recognized as a hydrophobic wax (135). The same material, or something similar, is also found at least partly covering the outer exposed walls of the mesophyll cells lining the substomatal chamber (60, 93, 145).

Stalfelt (157) recently reviewed the literature on cuticular transpiration and the voluminous literature on stomatal transpiration (158). Heath (66) and Kettelapper (78) very recently published reviews of the mechanisms of stomatal movement and the behavior of this apparatus in relation to the various environmental factors that correlatively affect it. These reviews should be consulted for details.

Evidence for stomatal control of water loss.—The role of stomatal closure in controlling transpiration has been difficult to determine because stomatal reactions are usually associated with a decreasing leaf water content. Most recent studies support van den Honert's hypothesis that stomata control transpiration, and hence, absorption is relatively little affected until the aperture is reduced to about half. He considers this to be true because closure would not be expected to gain control over water loss until stomatal resistance equals that of the gaseous phase itself. Others (9, 68, 178) consider that this observed "break point" occurs when interstomatal vapor diffusion interference disappears and each stomate loses vapor strictly according to the principles of Fick's law of diffusion for single pores.

It is considered probable by Slatyer (154) that in these laboratory studies the resistance to diffusion outside the leaf may be a major part of the total diffusion resistance in the gaseous phase. Under natural field conditions, on the other hand, leaf movement and turbulence reduce this external resistance component greatly (117). Then even the conductance of open stomates may be a significant controlling factor in water loss (136).

With decreasing leaf water content, Hygen (72) identified three phases in water loss from detached leaves of several species of mesophytes: (*a*) a constant-rate phase of water loss when little stomatal control is exerted, (*b*) a falling-rate phase when stomatal closure progressively reduces transpiration, and finally (*c*) a cuticular phase when stomates appear completely closed to an optical microscope with a one micron (diameter of 3000 water molecules) resolution limit. Superimposed upon this is the decrease in total water content that resembles the loss of water from porous media (103). This process, too, is characterized by an initial constant rate phase followed by an abrupt change into a rapidly falling rate phase as water content decreases.

There seems, therefore, to be a direct and indirect relation between leaf water loss and total leaf water content (73, 146). Gregory *et al.* (51, 52) and Milthorpe & Spencer (107) concluded that total leaf water *per se* is not important in the control either of leaf water loss or of absorption. If the falling-

rate phase begins at a critical total leaf water content, transpirational water loss will have far-reaching effects on the plant. Gregory *et al.* (51, 52), using single detached leaves of *Pelargonium* and *Triticum* in controlled-environment chambers with stomatal control eliminated by covering the leaves with vaseline, found that the falling-rate began when the turgid leaves had respectively lost 10 to 12 per cent and 5 to 6 per cent. Wide differences between plant types and times could be expected if only because of the differences in the effect of cell wall construction upon turgor pressure and water potential in the leaf. Until measurements of total water content are more precisely defined for geometrically smaller loci in the plant water systems, the coarser general correlation between total leaf water content and transpiration rate will have to suffice as a measure of the metabolic effects of internal water deficits.

The exact way in which total leaf water content at the critical falling-rate phase is related to a fall in transpiration rate is quite obscure. It is clear from studies on textiles (103), paper (159), and stomata (9) that lengthening the diffusion path from the evaporative surface decreases the conductance of the gaseous phase. We cannot expect, however, that the relation between leaf water content and transpiration rate will be quantitative in the sense that a certain leaf water content will always be connected with the same transpiration rate. Transpiration is not a static but a dynamic process (72, 98, 191).

Evidence for non-stomatal control of water loss.—A study of leaf anatomy and of the mechanism for opening and closing of stomatal guard cells has led many workers to suppose that stomata constitute the main or even the sole regulating system in leaf transpiration. Nevertheless, this supposition has been questioned on other grounds by many workers (17). The review by Maximov (103) of the arguments related to non-stomatal control of plant water loss is still valuable, and only more recent experiments cited in support of this hypothesis are mentioned here.

It is assumed by the incipient drying hypothesis that the wall contains a plethora of water-filled pores of various diameters. Under circumstances where the potential gradient from the leaf to the atmosphere is steep, the water in the capillaries retreats into the wall. This would (*a*) lengthen the gaseous diffusion path to the atmosphere and reduce vapor conductance, (*b*) decrease the water surface radius of curvature and reduce the capillary vapor pressure to that of the leaf water potential, and could (*c*) be connected with a reduction of water conductance of the cell wall.

The work of Meidner (104) seems to show that the vapor conductance of the substomatal cavities is appreciably altered by changes in the total leaf water content. The resistance of the mesophyll cells to viscous flow nearly doubles with a 6 to 10 per cent loss of water from cells initially turgid. The water conductance of the leaf tissue between the xylary termini and the substomatal chamber would decrease still further as leaf water content fell and intercellular spaces were more and more restricted. Milthorpe & Spencer (107) calculated that these changes in porometer-measured substomatal

conductances during leaf dehydration would be insignificant in wheat. The mesophyll resistance in *Pelargonium*, however, is a much larger variable component of total leaf resistance (65, 66), and this consideration could be very important. Species and plant growth history often may be very crucial in decisions of this type so that fortuitous selection of plant subjects may produce data of doubtful value as a basis for generalization.

The authors of the incipient drying hypothesis (94) did not detail the anatomy of the cell wall, since at that time little was known about cell wall microstructure. Since 1937, Frey-Wyssling (38) and others, using x-ray and electron microscope tools, have shown that spaces do exist between cellulose micellae. Their dimensions have been estimated. Recent work shows that the wall is much more complex than first thought. Waxes are found not only as a cell wall cuticle but are incorporated structurally (93) and may play a role in water loss control. The loss of water from the mesophyll cell must then occur *within* the cell wall layers, and the movement of the liquid phase to these water-losing micro-surfaces can occur only through a portion of the cell wall material.

Water molecules may follow two different routes from the vascular channels to the evaporative surface. They either may pass through cell walls and cytoplasmic membranes from cell to cell in the mesophyll or they may follow the submicroscopic inter-micellar channels in the cell walls and reach the surface without passing through any living cell. The studies of Strugger (166, 167) indicate that passage through the cell wall is by far the most important one quantitatively, although it is still not known to what extent these two pathways contribute to water transport under conditions of plant water stress.

Besides increasing the length of the water diffusion path to the outside atmosphere, and probably reducing the radius of curvature of the micro-capillaries of the cell walls, leaf water loss may also affect cell wall conductance. If the wall contains a micromosaic of cellulose, wax, and water menisci, and if water vapor can pass through only a small portion of the wall area, then cell wall contraction with decreased cell turgidity incurred by a leaf water deficit would reduce the area of the wall interstices, and water conductance would decrease. Perhaps the apparent lower transpiration rates observed during and after recovery from wilting are a reflection of some degree of hysteresis of the adjusting wall during rehydration. Preston (134) noted decreased water conductance of partially dried mesophyll cell walls. A decreased water potential (increased DPD) of mesophyll cells has also been associated with a greatly decreased permeability of mesophyll cell walls (15). Ordin & Bonner (118) noted that killing *Avena* coleoptiles does not alter their high permeability to D_2O. They suppose that this indicates that most of the resistance to water flow occurs in the walls.

A significant decrease in the water conductance of leaf cell walls may be important to plant water loss in cases where the water potential drop across the cell wall is appreciable in relation to the potential drop across the gaseous

phase between the wall and the external atmosphere (155), but we lack conclusive data on this matter. If cell wall cutinization were extensive, the water potential drop across it might be great, but the possible reduction of CO_2 and O_2 gas exchange might limit photosynthesis and growth.

Hygen's work (72) on detached leaves of various species produced curves of water loss rate indicating that the leaf drying process may proceed in two ways: by homogeneous drying and by surface desiccation. The homogeneous drying process would dominate when evaporation did not exceed the maximum conductance of the water-conducting channels. The relative water distribution in the tissues may remain unchanged, and a definite quantitative relation could exist between tissue water content and transpiration. If the diffusion capacity of the conducting channels is exceeded because of an excessive rate of water loss from the mesophyll cell walls, surface desiccation may ensue. Therefore, when internal water movement becomes a limiting factor in transpiration, the water content of the surface layer of the cell may decrease more and more independently of the water content of the interior tissues, which then in fact form an inaccessible water reservoir.

The term incipient drying (94, 103, 158) may therefore cover two types of drying processes, each conditioning transpiration differently. Hygen made it clear that his studies were conducted with only a moderate vapor pressure gradient from leaf to atmosphere, so that only homogeneous drying occurred. Therefore, his data apply to conditions where transpiration is a function of fresh weight.

Knight (80, 81) found that the transpiration rate of a *Eupatorium* detached-shoot with an internal water deficit as measured in a potometer was higher in a fan current of air, all other factors being equal, than in subsequent still air. The total water content of the shoot decreased. When the fan was turned on again the shoot gained water and the transpiration loss rose above that occurring when the fan had been on before. The rate of transpiration was thus related to shoot water content.

Although no direct measurements were made, these data have been used in support of the idea that the rate of leaf water loss when the fan was on resulted in "incipient" drying of the mesophyll cell walls. The walls rehydrated after the fan was turned off. When the air current was then begun again, water evaporated from these rehydrated walls and leaf water loss exceeded the rate when the fan had been on for a while. The walls again began to dry, and transpiration fell cotangent hyperbolically to the "normal" transpiration level when the fan had been on for a period.

Gregory *et al.* (51, 52) re-ran Knight's experiment, but with detached wheat leaves in potometers. They confirmed that the water content of the leaf increases during the still period, but could not disprove that incipient drying was no factor in water loss control. They did show that at least part of the higher transpiration recorded after the calm period was a result of increased stomatal aperture. It is worth commenting that neither Knight nor Gregory *et al.* used intact plants, and in neither case was an attempt made

to impose a tension on the xylem water column similar to that present when the potential gradient between the soil and the atmosphere is steep, but short of wilting the plant.

Measurement problems.—Difficulties are experimentally great in separating the probably predominant effect of stomatal control from the possible and probably minor effect of non-stomatal control and from cuticular loss of water. Stomatal control studies are aided by a water supply sufficiency at the root surface. Requisite conditions are full leaf turgor, fully open stomata, and a conduction rate at least equal to the transpiration rate. On the other hand, study of non-stomatal control of plant water loss would be aided by water deficit conditions. Desirable conditions are low soil moisture potential, open stomates, and a high rate of water loss, with transpirational loss exceeding the rate of water conduction and leaves approaching a wilted state. It is apparent that these two sets of experimental conditions are incompatible, and a direct factorial examination of the contribution of these two mechanisms of leaf water loss control is probably not possible.

Past studies attempting to assess the possible contribution of incipient drying to the attentuation of rates of water from leaves have suffered from failure to meet one or more of these conditions. In addition these studies have usually employed detached fragments of mesophyte leaf tissue (discs or shoots in potometers). Although in a few cases the gross loss of water from potometers has been treated on a rate basis over very short periods (107), almost nothing definite can be said concerning the intracellular behavior of water in an intact plant under conditions termed as moisture stress where either or both the soil moisture potential and atmospheric vapor pressure is low. We know only that plant water is redistributed internally and that total plant water content falls as the soil moisture potential decreases (149, 152) and approaches the permanent wilting percentage.

Only recently has some attention been given to the possible effects of gross plant water deficits upon plant cell particulates and their water balance (90, 174). From the use of D_2O and H_2O^{18} it has been concluded by these workers that cellular water consists of at least two fractions of unequal mobility and thus of unequal physiological value. The exact locus of these fractions, their mobility, and their relations to plant intracellular particulates are being investigated. A detailed study of the intracellular water activity of plants will doubtless furnish as much insight into the effects of plant hydration on growth as the study of cellular particulates did for our understanding of cellular metabolism.

INFLUENCE OF WATER DEFICITS ON PHYSIOLOGICAL PROCESSES

Problems of definition.—Studies of the influence of plant water deficits on physiological processes are hampered by several experimental difficulties. Particularly disturbing are problems of defining the experimental conditions. In studies of this type the definition of the degree of water deficit in the

treatment is still to be established. The concept of "relative turgidity" (187, 188) has helped, but since most of the water in growing higher plants in nature is in the vacuolated and other "non-living" regions this concept is not very sensitive as a quantitative measure of water deficits in the protoplasm. Reliable measurements of plant water potential are also difficult (153, 154) and the previous history of the plants further complicates the situation. Plants grown in nutrient solutions possessa different relative turgidity against water potential curves (189) than similar plants grown in soil. Plants subjected to high deficits during their life before experimentation will react differently than plants not experiencing such deficits (87). Lack of agreement on metabolic pathways in the absence of water deficits makes the detailed evaluation of results obtained during moisture deficits subject to the uncertainties of pathway interpretation. A good example of this is the influence of moisture deficits on photosynthesis where the uncertainty of pathways involved in the carbon cycle reflects on the conclusions reached (115, 116). Another difficulty worth mentioning is the choice of units for the expression of results. Weatherly (187, 188) and others (102) pointed out that water content of tissue expressed on a dry weight basis decreased during the growing season because of the increase in dry weight of the tissue with age. These changes occur in the absence of water deficits, and, because dry weight is not a constant base for comparison, it should be used with reservation. Gates & Bonner (43) have shown the difficulty involved in finding a truly unequivocal base for the expression of nucleic acid content in leaves under different water deficits.

Despite the difficulties in measurement and expression of results, there has been a revival of interest in recent years in the manner by which water deficits influence physiological processes and reduce measurable plant growth.

Protoplasmic hydration.—One of the more frequently cited reasons for changes in physiological activity under water deficits is protoplasmic hydration (73, 87, 92, 127). The importance of protoplasmic hydration is best exemplified in seeds where physiological activity increases very rapidly with increased water content (123, 124). Water deficits in the growing point, where a relatively larger volume of the cells are non-vacuolated, reduce growth rates appreciably (97, 169, 192). Plant hardiness, and specifically drought hardiness, seems to be related to the relative amount of bound water in the tissue; the more bound water in the tissue, the hardier the plant (92, 127).

Experimental evidence indicating the importance of protoplasmic hydration is ample, but one can rarely find a detailed evaluation of the mode of action involved. Since proteins are a major component of the protoplast, hydration of these proteins will take place and to a large degree will determine their spatial configuration. Klotz (79) concluded that protein molecules are hydrated with water envelopes and that the molecules of the hydration are arranged in a configuration similar to that of ice. He further suggested

that the structure of the hydration water can be markedly influenced by various factors, including change in the solute concentration of the medium. It may possibly be caused by changes in water potential. This change in the water potential, in turn, may result in reorientation and reordering of the protein molecules, and thus alter the relative distances between the enzymes that may be associated with this group of molecules. This could result in a modification of the enzyme reaction rates that offsets the "normal" balance of specific reactions and metabolites. The pattern of physiological activity of the tissue would therefore change. Skoog (148) showed that the nature of a tissue is correlated with the ratio between various metabolites in the cell. Changes in these ratios are usually observed under water deficits.

This cursory and hypothetical picture of the influence of protoplasmic hydration on growth processes was used in more detail by van Overbeek (122) in describing the mode of auxin action. He postulated that the association between the auxin molecule and protein causes a physiochemical change in protein hydration, thus altering reaction rates and the physiological pattern of the tissue.

Osmotic effects.—Turgor pressure has been given a major role in growth (87, 119, 120, 121). Several workers showed that the elongation of cells is correlated with the turgor pressure of the tissue (121). Theories concerning cell enlargement consider turgor pressure as a primary cause in the process, either through actual stretching of the cells or through increased water uptake by the cell as a result of increased extensibility of the cell wall and decreased turgor pressure (18 to 22; 67, 120, 121, 122). Ordin (121) recently attempted to establish whether decreased growth, particularly as expressed in cell wall development, was affected by the higher osmotic pressures of the cells under stress or by the decreased turgor pressure of the cells. He did this by studying the relative incorporation of C_{14} into various fractions of cell walls of *Avena* coleoptiles exposed to different water potentials obtained by pretreatment in mannitol and salt solutions. The reasoning was simply that the salt treatment should enhance the low water potential (high DPD) induced effects through absorption of ions if an increase in internal osmotic pressure is primarily responsible for high DPD effects. If, on the other hand, turgor pressure is the determining factor, the salt treatment should decrease the high DPD-induced effects, through increased turgor pressure. The data indicate that an increase in internal OP is not depressing in the metabolism of non-cellulosic polysaccharides; however, increasing OP values depress the formation of cellulose. Turgor pressure, in this study with *Avena* coleptiles, is the chief influence on both cell wall metabolism and elongation. It was shown that there is a parallelism between the response of elongation and cellulose synthesis to turgor pressure. On the basis of this and other observations, Ordin suggested that some aspect of cellulose synthesis may be involved in the elongation response to turgor. It is unknown why positive cytoplasmic pressure on the wall is needed for the maintenance of synthetic activities, but Ordin proposes some possibilities.

Cell division seems less affected by moisture deficits than is cell elongation. The data on this point are limited (87) but one indication is a comparison between cell counts on leaves grown under moisture deficits and those grown under adequate water supply. It was shown (103) that, although the stressed leaves are much smaller than the control, actual cell numbers are similar. Van Overbeek (122) suggested that the difference in response between elongation and cell division may be related to the rather higher requirement of elongation for oxygen.

Photosynthesis.—The relation between photosynthesis and water deficits has been studied extensively (2, 8, 16, 26, 34, 48, 113, 114, 168, 172, 179). In most of those experiments, however, water deficits were regulated by limiting the soil moisture supply to the plant, and only in some cases were there provisions for rigorous environmental control. Still fewer are studies that provide some description of the actual water deficits in the plant tissue itself(39). Therefore, the results do not always lend themselves to valid comparison.

Decreased CO_2 supply and a decrease in the diffusive capacity of the stomates are usually cited as the most common factors limiting photosynthesis under a given set of conditions. In addition to these a decreased water content in the leaves also causes decreased rates of photosynthesis, whether through decreased protoplasmic hydration or through the influence of these water deficits on stomatal opening and thus on their diffusive capacity.

Moss *et al.* (109) recently showed that relative responses to changes in CO_2 are much greater in photosynthetic rates than they are in transpiration. Under conditions near natural sunlight, net assimilation increased with increases in carbon dioxide above normal air concentration (0.03 per cent). They further showed that, on a sunny day, 95 per cent of the carbon dioxide would have to be supplied to an active corn crop by the atmosphere through turbulent processes. Heinicke *et al.* (63, 64) illustrated the dependence of photosynthesis on the speed of air flow over the leaves, implying that molecular diffusion of CO_2 to the leaf may limit photosynthetic activity. Several other studies and reviews indicate that CO_2 transfer into the leaf is one of the critical limiting factors for attaining potential photosynthesis in crop plants (39, 63, 179). Gaastra (39) recently attempted to evaluate the influence of light, CO_2, temperature, and stomatal diffusion resistance on photosynthesis. He concluded that, for his plants, 0.1 per cent was the CO_2 saturation concentration of photosynthesis. He further showed that, under low limiting light intensities, considerable stomatal closure may occur without affecting the rate of photosynthesis. Under light saturation and normal CO_2 concentration, however, the diffusion rate into the leaf determines the rate of photosynthesis. Stomatal opening, which is determined primarily by the water balance of the plant, is one factor controlling the rate of entry of CO_2 into the plant. Transpiration is more dependent than photosynthesis on stomatal opening and closing because transpiration depends on the algebraic sum of the resistances in the stomates and the surrounding air, whereas CO_2 entry depends both on these and on the resistance of the mesophyll. Gaastra's

results largely explain why experiments on the influence of water deficits on photosynthesis frequently yield conflicting conclusions. Studies under low artificial light yielded results indicating no appreciable change of photosynthetic rates due to plant water deficits (172). On the other hand, in different studies where photosynthesis decreased with increasing water deficits, light is probably not limiting (8). The literature contains papers contending that photosynthesis is not directly related to stomatal opening (172). A better relation is commonly observed between photosynthesis and water content of the leaves (26). As evident from the above discussion, it is difficult to make such distinctions but logical to expect a better relationship between water content and photosynthesis (just as between water content and transpiration) because the water deficit is a more inclusive variable than a stomatal opening alone.

Some observations indicate that the metabolic process of photosynthesis is less affected by water deficits than is growth by elongation. *In vitro* studies of photosynthesis use rather high concentrations of sugar in the medium and though the concentrations may be equivalent to over 10 to 15 atm. of osmotic pressure, photosynthetic rates seem to continue unimpaired. Cell enlargement or elongation under such water potentials stop completely or continue at a slow pace after a period of readjustment. Gingrich & Russell (46, 47) have shown that growth of the tissue as measured by dry weight is less affected by water deficits than is elongation. Other workers (58) made the same observation.

Several workers (2, 8, 144, 172) showed that photosynthetic rates decrease rapidly or approach zero when wilting begins. Furthermore, the recovery of original rates after re-irrigation took several days (8). Similar results have been obtained for transpiration (85). This indicates that the inhibitory effects of internal water deficits upon photosynthesis are intimately related to the water balance of the tissues through the effects that such deficits may have on diffusion rates into and out of the leaves and through the possible effects of changes in protoplasmic hydration under these conditions.

Plant composition.—Many papers report on the specific changes in composition and metabolism that take place in plants under a water deficit economy. Much of the available data at present do not lend themselves to detailed interpretation of the manner by which water deficits influence these shifts in metabolism and the biochemical composition of the tissue. Some reactions are more sensitive to moisture deficits than are others (115, 120, 121). When a sensitive reaction is affected, it is reasonable to assume that a general shift in metabolism will develop gradually. One such reaction is cellulose synthesis, which seems to depend on turgor pressure (120).

Some of the earliest work concerned the influence of water deficits on carbohydrates. The accelerated conversion of starch to sugars during water deficits has been observed by many workers (32, 100, 156, 181, 195). It has been shown that increased amylolytic activity is involved in the disappearance of starch. Generally, the rapid starch reduction is not compensated for

by corresponding increases of sugars (195), suggesting that respiration rates might have increased because of the water deficits. Several investigators found that to be the case (111, 129, 144, 172, 197). Schneider & Childers (144) showed that flaccid leaves respired 62 per cent faster than the leaves of turgid control plants, and that the activity of oxidative enzymes is accelerated in stressed plants. Data have been obtained for polyphenol oxidase, ascorbic acid oxidase, catalase, peroxidase (129, 132, 170, 196, 197), and proteolitic enzymes (110). Yarosh (196) found that the respiration of cotton changes markedly in response to changes in water supply and he suggested that catalase and ascorbic acids are good respiration indices in the cotton plant. This conforms to conclusions by Toschevikova *et al.* (171).

Several studies have been carried out on the relationship between water deficits and protein metabolism. Mothes (110) and other workers (196) showed a relationship between water content and proteolysis. Proteolytic enzymes are activated by reduced sulfhydryl groups, and their activation may be enhanced because of oxygen deficiency resulting from stomatal closure (110). Petrie & Wood (129) and Yarosh (196) observed that the net formation of proteins from amino acids decreased as moisture deficits increased. Yarosh reported the ratio of non-protein to protein nitrogen was between 0.14 to 0.24 in unirrigated cotton and 0.11 to 0.19 in irrigated cotton. He attributed this to decreased synthesis and increased hydrolysis. Ivanov (75) recently showed that amino acid synthesis decreased under moisture deficits as determined by chromatographic analysis of guttation fluid. Gates & Bonner (43) showed that the amount of both DNA and RNA per leaf decreased under water deficits, although they were similar in the water-deficient and control treatments of tomatoes on a dry weight basis. The decrease in nucleic acids per leaf is intimately related to the slower growth rate per leaf. Gates and Bonner attempted to verify whether this decrease was due to decreased synthesis in response to water deficits. Their results show the rate of P^{32} incorporation into nucleic acids under moisture deficits remaining unchanged with respect to the control. This indicates that the decrease in net production of nucleic acids under water deficits is probably due to accelerated destruction rather than decreased synthesis. Since protein synthesis is related to RNA, the effects of a moisture deficit on nucleic acids will reduce the rate of protein synthesis indirectly.

Reports on the biochemical composition of tissues under moisture deficits are frequently conflicting. For example, Haddock (56) and Maksimovich *et al.* (102) reported a lower sugar percentage under moisture deficits in sugar beets at harvest time, whereas Wiersma (190) reported a higher percentage. Such differences, however, are probably attributable to other variables such as fertility rather than to moisture regimes. In general, the per cent nitrogen in grain is observed to increase under deficits (82, 128, 197). Konovalov (82), for example, found that the grain in stressed wheat plants contained about 2 per cent more nitrogen than in control wheat. This increase in the grain results from increased proteolysis in the vegetative parts and translocation of

the resulting compounds to the developing grain. Zholkevich *et al.* (197) showed that translocation of material to the wheat grain is accelerated during a water deficit. It is generally observed that the nicotine content of tobacco leaves is significantly increased under moisture stress (11, 25) while other alkaloids, in contrast, appear to decrease (99). The rubber in guayule is reported to increase with water deficits (71, 184). These observations are important to the agriculturalist for they may dictate his irrigation practice for the best quality of crop. Plants in an enhanced state of maturation and under severe moisture deficits develop symptoms that can be interpreted as senescence (40, 41, 42). No detailed interpretation of the effects of water deficits on plant composition is yet available.

Stage of growth.—Although plant growth is reduced under internal moisture deficits, this decreased growth may frequently be recovered by applying water. Gates (40) showed that, after the removal of water deficits, the rate of elongation of tomatoes was faster than in controls. Similar observations were made by Owen (125). If irreversible damage can be caused by water deficits, however, such irreversible influence at any particular stage may affect subsequent growth rate and total yield at maturity. Maize is one of the crops on which we have some information in this regard. Robins & Domingo (143) showed in field experiments that abundant moisture is essential during the tasseling stage for maximum corn yield. Water deficits during that period cause an irreversible decrease in yield. Denmead & Shaw (28) showed that moisture stress at silking reduced yields by 51 per cent. They suggested that moisture deficits during vegetative growth have only an indirect effect on grain yield by reducing the size of the assimilatory surface (6, 7) at the time of ear development. By contrast, deficits after ear emergence have a more direct effect through reducing assimilation in the critical period when daily assimilation rates are high and when most of the assimilate is being used in grain production. It was shown (74) that flowering is a critical growth stage for peanuts. Crop yields from plants abundantly supplied with soil moisture at this stage are equal to those of plants abundantly supplied with water at all stages of growth. Critical growth stages have also been shown for wheat and barley (31, 82), castor bean (89), clover (58), and other crops.

Moisture, through its influence on the metabolic rhythm of plants, may exercise some control on the flowering response of some plants. A good example is the "moisture deficit requirement" of coffee, a short-day plant. Mes (105) suggested that water deficits kept the buds dormant and that the involved inhibitions are released by removal of the deficit. Alvim (3), however, suggested that water deficits are not the cause of dormancy but the cause of breaking the dormancy. This conclusion is based on the fact that in different irrigation treatments buds remained dormant in wet treatments but flowered after irrigation in the dry treatments. Irrigation, or rather, lower water deficits are therefore necessary for the resumption of growth rather than for the initiation of flowering.

Nutrient uptake.—The relationship between nutrient uptake and water

uptake is reviewed elsewhere (142). We shall merely comment on certain observations concerning the effects of moisture deficits on nutrient uptake. The experimental observations are rather equivocal. A recent review (61) listed nine papers reporting that P uptake was unaffected within wide limits of soil moisture contents, and twelve papers reporting that P uptake is decreased with decreasing soil moisture. Since these investigations involve complicating factors contributed by the soil, and since no evaluations of plant water deficits are cited, it is difficult to conclude much from these data on the relationship between nutrient uptake and water potentials. Nevertheless, there is an indication in some papers as to the water deficit imposed (24, 27, 41, 42, 68, 91, 101). These seem to show decreased nutrient uptake with increasing water deficits for phosphorous, potassium, and rubidium. The reasons for this decrease may depend on binding of the salts in the soil (184), or on decreased soil water conductivity (24), or on metabolic effects (62).

Philip (131) suggested that at low moisture contents in the soil a vapor gap may be formed around the roots by their decreased turgor under deficits. Such a gap, if present, would reduce the availability of nutrients to the roots. Philip's idea has been contested on theoretical grounds (12). Nevertheless, although the existence of a vapor gap is questioned, the contact between roots and soil solution is probably not as intimate under low soil moisture content.

The manner in which active accumulation is affected by water deficits and the magnitude of these effects are unknown.

RELATION OF PLANT WATER DEFICITS TO SOIL MOISTURE AVAILABILITY

The concept of moisture availability.—A vigorous controversy has continued for a number of years on whether the so-called "available soil moisture" is equally available for plant growth between field capacity and the wilting point or whether this water is taken up with such increasing difficulty that plant growth functions are retarded before the wilting point is reached. These viewpoints are summarized in several recent review articles (76, 138, 151, 175, 177). The statements under controversy attempt to relate soil moisture to plant growth, usually excluding measured and unmeasured plant or climatic factors that are necessarily involved in studies of water-soil-plant relations.

As discussed earlier the evidence suggests that water deficits within plant tissues check growth and that the degree to which growth is checked depends on the relative deficits developed within the plant. Internal water deficits are not necessarily related directly to soil moisture. Rather, they depend at any given time on the balance between water lost from the shoot and that absorbed by roots. Whenever loss exceeds absorption, some deficit develops. Although internal water deficits are everyday occurrences in crop plants, water absorption at night usually offsets the deficits produced during the day, permitting recovery of turgor and continuance of what is recognized as normal growth. This normal growth is probably not the maximum growth of

which the plant is potentially capable (30). If water deficits developed in plant tissues within diurnal cycles are not restored during the night, then progressive decreases in growth should be observed.

When attempts are made to establish a relationship between soil moisture parameters and plant growth, we assume that there is a unique relationship between internal water deficits in the plant and these soil moisture parameters. This, however, cannot be universally true. Since water deficits within plants depend on the balance between water loss and water uptake (the relations between demand and supply), soil parameters alone cannot be directly related to plant water deficits. Such a relation would be modified by the factors that create the demand for water on one hand, and control the supply on the other. Among these factors we should recognize the climatic conditions prevailing and creating the evaporative demands, the type of plant, its stage of development, and its root system. When evaporative demands are high, a given rate of supply may become inadequate. When absorption lags behind loss, water deficits develop within the plant and water potential gradients steepen, tending to accelerate water absorption through the roots. Also, the plant itself makes adjustments to the situation, frequently through a control on its losses by stomatal closure or other means. This decreases transpiration rates, which slows down the development of appreciable internal water deficits. Thus, whenever transpiration rates decrease to values below those permitted by the prevailing environmental demands, water deficits must be increasing progressively within the plant.

The proponents of "equal availability" of soil moisture for plant growth between field capacity and wilting point (175, 176, 177) imply that the rate of water supply to plant roots is not limiting to plant growth between these so-called soil moisture constants. The proponents of an inverse relation between soil moisture suction and plant growth (138, 182, 183) suggest that an increase in soil moisture suction will affect the internal water deficits of plants and reduce growth proportionately. The following illustrations show that each viewpoint is a special case, not of universal validity.

Soil moisture considerations.—Field capacity and the wilting point are still useful concepts since they indicate approximately the capacity of soil to store water for plant growth. Even so, they should not be regarded as constants but rather as somewhat arbitrarily defined ranges of soil moisture. Although "available moisture" has been defined as the difference between field capacity and wilting point, plants may use considerable amounts of water from soils having moisture contents above field capacity or possibly some water below the wilting point (149, 150). Because of this, the commonly used expression of "available soil moisture depletion" may be in error. Slight discrepancies in the values chosen for field capacity and wilting point will materially influence the quantity of water apparently held within the "available range." Thus, depending on the accuracy of the values of field capacity and wilting point, one may draw conflicting conclusions on the relation to plant growth within the available range.

Peters (126) set up an experiment that illustrates the difference in effects on plant growth between soil moisture potential and soil moisture content. He mixed clay and sandy soils in various proportions to provide mixtures capable of retaining a wide range of water contents at given suction values. He covered germinated corn seeds in controlled-growth chambers with the various mixtures of soil, previously brought to definite soil moisture potential values. The results show that plants at the same potential levels grew better in the finer than in the coarser soil. That is, water absorption was controlled by both soil moisture potential and moisture content. The greater hydraulic conductivity of the finer soil is probably the primary cause of the better plant growth observed. This illustration further points out some of the difficulties inherent in developing a universal relationship between growth and soil moisture potential or plant water deficits.

From the theoretical standpoint, it is important to eliminate such variables as fertility in soil moisture studies. In field experiments, however, this is rarely possible, for the fertility levels of agricultural soils may vary considerably under different moisture treatments. In soils deficient in nitrogen, yields increase greatly with fertilizer application, and but little with water applications. If a relationship is to be established between soil moisture regime and growth, it would be different at different levels of fertility. The relative dryness of the soil does not decrease yields as much at low nitrogen levels as at higher nitrogen levels (59).

One could cite several other examples illustrating ways in which fertility may influence an attempted correlation between soil moisture and plant growth. From an experiment with corn seedlings growing in osmotic solutions and in soil samples with equivalent moisture suctions, Danielson & Russell (24) concluded that the rate of accumulation of Rb^{86} was not directly affected by increasing osmotic concentrations in solutions but was reduced with increasing soil moisture suction. They suggested that the cause may be a reduced rate of water movement to the roots from the decreased unsaturated conductivity of the soil. Over the course of a growing season these decreases in nutrient uptake from the relative dryness of the soil may influence plant growth as much as water uptake itself.

Plant considerations.—Plants have been classified ecologically as to their water relations and their ability to survive water shortages (103). Under similar environmental and soil moisture conditions, plants that can control their water loss efficiently, develop a well-branched root system, and develop high internal suctions, will not be affected in growth as much as plants lacking some of these characteristics.

Some plants appear capable of utilizing water more efficiently than others (29, 30). One factor contributing to this may be a difference in the abilities of plants to control their water balance. A generalization concerning growth as a function of soil moisture conditions cannot include considerations of this type and would therefore be lacking in general validity.

Another factor of extreme importance in determining the relationships

between plant water deficits, growth, and soil moisture is the nature of the root system. The root system provides the surface for absorption of water by the plant, and therefore can exercise marked influence on the balance between lost and absorbed water. The greater is this absorptive surface, the smaller is the likelihood that water supply will limit plant growth. Root systems rarely have uniform distribution with depth. This nature of the root system makes generalizations on the relationship between soil moisture and plant growth very difficult. The non-uniformity of rooting density within the root zone, the unknown fraction of the total absorbing roots in deeper layers, and, of primary importance, the continued growth of roots into moist soil particularly in annual crops, make it very difficult to evaluate the actual soil moisture potential to which the plant is subjected. A further complication arises because root distribution itself is materially affected by soil structure and fertility. This makes it difficult to compare results from different areas, even with a single crop.

We recognize several different manifestations of growth, such as elongation of plant organs, increase in fresh or dry weight, and vegetative versus reproductive aspects. These processes are a result of the intricate combinations of many physiological processes, and not all of them are equally affected by water deficits and any accompanying changes in the cells and tissues, as shown previously. The reduction in fresh weight from moisture deficits may be proportionately much greater than loss of dry weight (47, 58). With certain kinds of crops moisture deficits during maturation are not harmful, and in some cases may be beneficial. An example is a crop grown for seed (58) and other portions of the reproductive organs, such as cotton (164). During the seed or flower maturation period, the vegetative growth in these crops may be drastically reduced by moisture deficits without a measurable decrease in yield of the harvestable organs. Even in these cases, however, moisture deficits earlier in the growth period will reduce vegetative development and, ultimately, the yield of harvestable organs (58).

The fact that various measurable aspects of growth do not respond in the same manner to water deficits further complicates the problem of formulating a simple relationship between soil moisture and plant growth, thus making any sort of generality along these lines difficult.

No specific examples of the type given above are noted here for atmospheric or climatic variables. Such discussion can be found elsewhere (13, 29, 59). However, climatic variables just like those involved in the soil and the plant will exercise control over the water balance of plants in a manner that cannot be predicted without basic information regarding the flow characteristics of the whole system.

CONCLUSIONS

Plant growth does not depend directly on soil moisture supply but rather on the balance between transpired and absorbed water. This balance depends on soil, plant, and climatic factors. Prediction of water deficits cannot be

made from soil moisture data only nor can it be predicted from transpiration data alone. Relationships developed between soil moisture content or soil moisture potentials and plant growth will of necessity be applicable only in an approximate manner to the crop under the prevailing soil climatic and management conditions. Relationships of this type should not be expected to provide basic principles of wide application, but rather to furnish the basis for irrigation recommendations useful only in given situations.

Future efforts in plant-soil-water relations should center around the physical and biochemical aspects of the continuum. Our emphasis should be directed towards perfecting techniques for measuring plant water balance and evaluating its relationship to the evaporative demand on the one hand and to the supply on the other. Flow characteristics of water in the soil-plant-atmosphere continuum should be evaluated in order to gain better understanding of the development of water deficits. Greater emphasis should be given to the relation between water deficits and metabolism, and the manner by which decreased water potentials influence reaction rates and induce shifts in the physiological patterns of the tissues. Only then will we be able to formulate a universally applicable concept of the dynamic relationships between water and plant growth.

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PHOTOPERIODISM AND THE FLOWERING PROCESS[1,2]

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INTRODUCTION

Several excellent reviews on the physiology of flowering have appeared within recent years, four of them in these volumes. The review of Lang (100) initiated the series and was one of the most complete, covering both photoperiodism and vernalization. Liverman's review (111) and that of Doorenbos & Wellensiek (48) emphasized photoperiodism, and Chouard's (43) comprehensive review of vernalization appeared in the last volume. Naylor has reviewed the subject extensively (139, 140, 141). His last review and a companion one by Lockhart (114) were in press at the time of this writing. Many other summaries have also appeared (e.g., 10, 11a, 102, 119, 130, 132, 138, 148, 167, 205, 209, 212). Recently a collection of papers presented at a symposium entitled *Photoperiodism and Related Phenomena in Plants and Animals* (217) has been published. These papers are both general and specific in nature. The topic of photoperiodism has also been discussed at a number of national and international meetings (e.g., 42, 73, 91).

Because of this profusion of recent excellent reviews, complete coverage of all of the literature should not be essential, and Chouard's (43) thorough discussion of vernalization obviates the necessity of including this topic. Papers that contribute rather directly to the theory and understanding of flowering in response to photoperiod are emphasized. In many cases only representative papers are cited although others have been published that support the topic. The approach is similar to Liverman's (111) in that the component processes of the flowering response in short-day plants (especially *Xanthium*) are emphasized. Since his review, however, increasing emphasis has been placed upon the general significance of these processes to biology (note the above mentioned symposium). Virtually all the steps of the flowering process have their counterparts in other plant and animal functions; photoperiodism implies a response to light and a measurement of time, both of which phenomena are now known to be widespread (measurement of time may prove to be a characteristic of living things). Synthesis and translocation of the flowering hormone are less general in nature, but response to the hormone is an example of what might be the most fundamental phenomenon of biology: differentiation or morphogenesis. And if morphogenesis is commonly a response to specific chemical substances, then knowledge concerning the

[1] The survey of literature pertaining to this review was concluded in October 1960.

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synthesis, movement, and mode of action of the flowering hormone is certainly of basic biological significance. The following discussion considers these fundamental problems as they apply to the flowering process. Topics such as the pigment system and measurement of time are discussed as they apply to plants in general, but space limitations seldom allow any mention of how these basic processes operate in the various specific long- and short-day plants that have been studied.

GENERALIZATIONS ABOUT THE FLOWERING PROCESS

The details relating to flowering are extremely involved and complex, and contradictions are not uncommon in the mass of literature that has accumulated on the subject. As J. Bonner (8) has said, "Perhaps it is impossible for one individual even to remember simultaneously all the relevant facts." Thus the basic facts should be summarized before recent developments are considered. To this end eight generalizations have been formulated. Obviously, future research may prove them to be less general than is implied here, but at present they appear to be the "typical" responses of the plants that have been most intensively studied. Choice of what to include must be a very arbitrary matter, and the author assumes complete responsibility.

I. Short-day plants flower (on a 24-hour cycle of light and darkness) if the day-length is less than some critical amount. Experiments utilizing cycles greater or less than 24 hr. have shown that short-day plants flower more in response to dark periods that exceed a minimum critical length than to the length of the light period. The light and dark periods may interact, however.

II. Long-day plants flower in response to day-lengths exceeding a certain minimum critical value (cycle lengths of 24 hr.). Again, the response to dark periods of less than a maximum value appears to be more critical than the response to the light period, and an interaction may complicate this situation.

III. Short-day plants are inhibited and long-day plants are promoted in their flowering by an interruption of the dark period with light.

IV. The action spectrum for light interruption of the dark period in both short- and long-day plants shows a broad peak in the orange-red between 620 and 665 mμ (12, 149, 150), and the effects of this orange-red light are reversed by far-red light (peak between 710 to 740 mμ) (13). These action spectra place the light interruption responses in the realm of other light phenomena in plants which are controlled by the so-called photomorphogenetic pigment system (217). Effects of blue are also encountered in these systems as well as in flowering (125, 126, 127).

V. There is strong evidence (see below) for a flowering hormone (stimulus) that moves from the leaves to the apical meristems. This hormone has been called florigen (39).

VI. Flowering in the majority of photoperiodically sensitive plants is an inductive process. That is, plants given the photoperiodic treatment that promotes flowering will continue to produce flowers even though they are returned to a photoperiodic regime that would not lead to flowering. One or

more, and sometimes many, photoperiodic cycles are required to bring about this condition. (See introductory consideration of the induced state, below.)

VII. A combination of responses is commonly observed within a single plant. Many plants are known (184) in which the response may be day-neutral at one temperature and sensitive to day-length at another temperature; species are being discovered that require a combination of day lengths in a particular order; and a few plants are known to respond to light intensity changes (96, 177, 185, 187). Indeed, plants are relatively rare in which the day-length response is only slightly influenced by temperature. Even the test plants of photoperiodism studies are so influenced to a greater or lesser degree: *Xanthium pennsylvanicum* Wall. (cocklebur), *Glycine max* (L.) Merr. (Biloxi soybean), *Hyoscyamus niger* L. (henbane), *Perilla ocymoides* L., *Chenopodium amaranticolor* Coste *et* Reyn., *Kalanchoë blossfeldiana* v. Poelln., etc. Recently, a few others have been added to this group. Of these, *Pharbitis nil* Chois. (Japanese morning glory) is the most important. It is a very sensitive short-day plant that responds to a single inductive dark period. Seedlings are usually used, and the characteristics of their response have been worked out in careful detail by the Japanese (85). *Pharbitis* has recently been shown to be day-neutral at low temperatures (144, 201) if plants are given sucrose (191). *Lemna perpusilla* Torr. 6746 (82, 83) is also of special interest because experiments with this plant may be carried out in only a week and its response to day-length is influenced by its nutrient medium (it responds to a single dark period but only when grown on media containing iron chelating agents).

Plants that require short-days followed by long-days were described as early as 1930 by McKinney & Sando [winter wheat (123)] and again by Loomis and co-workers in 1949 [certain grasses (63, 151)]. Resende (157) and others [see discussion in (161)] have also described such plants, as well as those that require long-days followed by short-days (49, 156). The present interest in short-long-day and long-short-day plants, however, stems from the detailed work of Sachs on *Cestrum nocturnum* L., a woody plant (159, 160, 161). Among other things, Sachs showed that light interruption of the dark period inhibited the short-day phase of induction and promoted the long-day phase, as in plants with only a single day-length requirement. He suggested (159) that all plants have a series of requirements for flowering that, in the dual day-length plants, may be met only by the proper sequence of day-lengths. For example, a "normal" short-day plant may require a "long-day" of only two or three hours preceding the short-day phase of induction. Considering the long-short- and short-long-day plants together, he concluded (161) that one should not expect the results of long-day treatment to be metabolically the same in all long-day plants, nor of short-day treatment in short-day plants. Considerable evidence is accumulating in favor of this suggestion (48, 114).

VIII. In short-day plants a number of component processes have been recognized (48, 111, 166, 171). High-intensity light must precede the dark

period if the latter is to be effective in inducing flowering. Timing mechanisms in the leaf that involve an internal conversion of the photomorphogenetic pigment from one form to another, and probably other unknown mechanisms, constitute the first part of the dark period. The second part of the dark period is concerned with a synthesis of flowering hormone. This hormone must then be translocated from the leaf to the apical or lateral meristems or both; these respond by producing floral organs instead of leaves. In long-day plants the component processes may be similar, but evidence for their existence has been difficult to obtain because of certain inherent properties of the plants that are normally used. Most of the recognition of the component processes in short-day plants depends upon work with *Xanthium*, frequently used because it responds to a single inductive cycle. Only recently have long-day plants of such sensitivity been described (56) and as yet comparable work has not been done with these plants, although the component process concept is being extended (57).

THE PIGMENT SYSTEM

Three principal questions relating to the photoreceptor pigment will be considered. The first question concerns the nature of the pigment; the second, the kinetics of its action in the flowering process; and the third, the biochemistry of its action in flowering.

What Is the Pigment?

Since light must be absorbed to bring about a photochemical reaction, a pigment system must form an integral and essential part of the flowering mechanism. Action spectra (generalization number IV) indicate a photoreversible pigment system that may be represented as follows:

$$\text{R-Phytochrome} \underset{\text{Far-Red (730 to 735 m}\mu\text{)}\ \text{(or more slowly in darkness)}}{\overset{\text{Red Light (660 to 665 m}\mu\text{)}}{\rightleftharpoons}} \text{F-Phytochrome}$$

R-Phytochrome refers to pigment in the red-absorbing form, produced by illumination with far-red light or equilibration in darkness. F-Phytochrome is the far-red-absorbing form of the pigment. A prime object of research has been the isolation and characterization of this system. Much of the physiological work has been carried out by workers at the Plant Industry Station at Beltsville, Maryland [including Hendricks, Borthwick, and others (11a, 13, 50, 80, 81)]. It is appropriate then that Butler *et al.*, working with this group, were the first to succeed in obtaining *in vitro* preparations of the photomorphogenetic pigment system (11a, 27, 28, 29).

The reversible nature of this system suggested an assay. Absorption peaks of the pigment should be dependent upon the quality of light last used to illuminate the pigment. The obvious difficulty lies in the fact that light used to measure optical density at either absorption peak would tend to destroy that peak. The Beltsville group solved this problem with a spectrophotometer system possessing high sensitivity and capable of measuring

transmission through dense samples. A reaction-forcing illumination of bright light (e.g., red) is given, and optical density at the desired peak is measured. Optical density is again measured following a second reaction-forcing illumination with the "opposite" wavelength (e.g., far-red). The difference between the two optical densities is a measure of the presence and quantity of the pigment system. The original equipment was assembled from component parts at Beltsville, and commercially available equipment has also been used (6). Thus work with the pigment system can be carried out in any laboratory having sufficient interest.

The Beltsville workers have obtained the pigment *in vitro* from a number of dark-grown plant tissues. Corn seedlings have proved to be a rich source, and B. Bonner (6), in another laboratory, has used etiolated pea tissue. The pigment has been obtained at Beltsville from green spinach leaves, but the presence of chlorophyll complicates the procedure.

The Beltsville workers named the pigment system "phytochrome." It is of considerable interest that phytochrome proved to be protein in nature since chlorophyll is thought to be closely associated with protein in the chloroplasts, and the phycobilin pigments must also depend upon protein for their photoactivity. Initial attempts to separate a prosthetic group from phytochrome have been unsuccessful. Indeed, attempts have been sufficiently extensive to permit the tentative conclusion that the chromatophore is not a dialyzable group. Complete purification of the pigment system had not been accomplished at the time of this writing although its concentration *in vitro* had been increased at least tenfold. At any rate, study of the photoreceptor pigment system *in vitro* has opened a great many doors to our potential understanding of photomorphogenetic processes in plants.

What Are the Kinetics of Action of the Pigment System in Flowering?

The deductions based upon experiments with whole plants now await confirmation in the isolated phytochrome system. An elegant series of experiments by the Beltsville workers led to the conclusion that the photoreaction was first order in both directions (81). This has been confirmed in the *in vitro* system (28, 29). Another early conclusion of these workers, that the conversion of the pigment from one form to another involved a reaction (probably oxidation-reduction) with another compound (e.g., 79), was not substantiated in work with the *in vitro* pigment (6, 28, 29). However the majority of the early conclusions, including the idea of reversibility itself, were substantiated. Yet those of us who indulge in the essential practice of deducing ideas about the chemical nature of the flowering mechanism from the results of physiological experiments with whole plants, should again be reminded that these conclusions must remain tentative until they are supported by more direct evidence.

A key problem, discussed rather extensively by the Beltsville group (80), concerns the action of the pigment when illuminated by relatively high intensity light which contains various ratios of red and far-red. They demon-

strated that a number of photomorphogenetic responses could be understood if this problem was properly taken into account; and it is also of importance in flowering (see below). Recently, they have shown that under incandescent illumination (slightly more far-red than red) about two-thirds of the pigment consists of F-phytochrome. Thus it is significantly easier to drive the reaction from R-phytochrome to F-phytochrome than it is to drive it in the reverse direction. Stated another way, red light is more effective than far-red light at equal intensities (R-phytochrome has a higher absorption coefficient than F-phytochrome; quantum efficiencies are near 1.0).

The workers at Beltsville (28) as well as Bonner (6) have studied the destruction of the pigment *in vitro*. The conversion of F-phytochrome to R-phytochrome in the dark does not occur *in vitro*. The Beltsville group has also studied its synthesis and conversion *in vivo*. Present results are too preliminary to permit any far-reaching conclusions, but developments in this field are anticipated.

Although we are just beginning to learn about the kinetics of the light response *in vitro*, considerable research has been done with whole plants. The studies on germination, dormancy, stem extension, leaf expansion, and other photomorphogenetic responses are probably pertinent (217), but space limitations will not allow a discussion of this work here. A few general principles will be discussed as they relate to the flowering process.

Reciprocity.—Withrow (218) has considered the question of reciprocity. If a photochemical reaction is stoichiometric, only the quantity of light is important. That is, light of high intensity applied over a short duration should be equal in effect to the same quantity of light applied at lower intensity over a longer duration. Withrow has pointed out that this reciprocity relationship seldom holds in photobiological processes, since short duration times are relatively more effective than longer times. (Reciprocity failure is also common in photochemistry—including photography.) We might conclude on the basis of relatively early work in photoperiodism that reciprocity does not fail in this process (12, 149, 150). Since that work, however, the methods used in flowering research have been improved so that smaller differences in response may be observed. In the early work, results were expressed as a percentage of the plants treated alike which ultimately flowered. Thus, expression of the data assumed nonuniformity of the material. If the uniformity of response were extremely high, either all plants would flower or none would flower, and percentage data would have little meaning. During the past decade most workers have overcome this difficulty by expressing their data according to some measure of the degree of flowering. Early workers did not use this method because it was felt that conversion to the reproductive state was the most important aspect of flowering, and confusion with development was to be avoided. Yet the rate of development is strongly dependent upon conditions during induction (the dark period in short-day plants) and relatively refractory to the usual conditions during development (164, 165). All plants in an experiment develop under the same con-

ditions anyway. Sometimes these latter conditions will cause borderline stages to revert to the vegetative state (165). In a percentage system these early stages contribute as much to the per cent flowering as the more advanced stages, but in a stage system the numbers assigned to the low stages are usually in accordance with their borderline nature.

The problem of reciprocity in flowering has yet to be studied in sufficient detail and by use of proper methods. The experiment should be done with an accurate control of light (intensity, quality, and duration) and using highly uniform plants trimmed to a single leaf (to avoid problems of shading and the sensitivity changes dependent upon age); the light interruption should be applied at the proper time during the dark period (discussion below); and data should be expressed using a system of floral stages. In a classroom demonstration the author obtained results, using an electronic flash (designed for photography), that tentatively show reciprocity failure in flowering. It has also been reported that reciprocity fails in *Pharbitis* (199), but in the opposite sense to that mentioned above.

The time of effectiveness of an applied light interruption.—This has been a subject of experimentation by many workers during the past two decades (e.g., 48, 95, 100, 111, 171). Recent work has been done with dark periods of relatively long duration, but since this is probably more closely related to the question of timing than to the kinetics of the pigment system, discussion will be postponed until the next section. In general it may be concluded that maximum inhibition of flowering in short-day plants (or promotion in long-day plants) occurs when the light interruption is applied near the end of a duration of darkness nearly equal to the critical dark period. With "normal" dark periods (within a 24-hour cycle) maximum effectiveness often comes near the middle. The per cent flowering should be influenced only by light interruptions that divide the dark period into periods close to the critical, and this is generally the case. Thus, experiments on time of effectiveness of a light interruption must use a quantitative measurement of flowering. If the numbered stages can be shown to have any sort of linearity, kinetic considerations of the data will be more reasonable (165, 173).

Effective quantities of light used in interruptions.—This problem has also received considerable attention (48, 100, 111) since it could indicate the quantity of photoreceptor pigment present in the leaf as a function of time during induction. Unfortunately, many of the results are conflicting and difficult to interpret. There are at least three immediate reasons for these deficiencies. First, before any work of this nature can be meaningful, the question of reciprocity must be resolved. Second, many of the data have been expressed on a percentage flowering basis which is subject to the difficulties mentioned above. Third, it is quite conceivable, and results seem to indicate (171), that it may not be necessary to convert the pigment system completely to F-phytochrome to cause complete inhibition of flowering during the most light-sensitive portion of the inductive dark period. A fourth, and probably equally important, point is that it is experimentally difficult to

control the quality, quantity, and duration of light that simultaneously reaches the leaf surfaces of all the test plants in a given treatment.

Nevertheless, experiments with *Xanthium* (171) in which intensity was held constant and duration was varied seemed to indicate that the amount of light required for maximum response at a given time (saturating quantity) remains constant during most of the inductive dark period. From these results it was concluded that the photoreceptor pigment completed its conversion to R-phytochrome within the first two or three hours of the inductive dark period, and that the amount of R-phytochrome remained relatively constant during the rest of the dark period. Könitz, in Bünning's laboratory (21, 24, 95), came to opposite conclusions (using *Chenopodium*). He reports that red light interruptions during the dark period could always produce vegetative plants, but that the amount of light required varied drastically during the dark period—the least amount near the middle, much more towards the beginning or end. It was concluded that the amount of R-phytochrome changes as drastically during the inductive dark period. This conclusion is, however, subject to serious question. Since Könitz's plants were not defoliated to a single leaf, one might expect shading and differing sensitivities among the various leaves (93, 171) to make it virtually impossible to obtain a saturation value. Furthermore, saturating quantity cannot be determined if the interruption causes plants to remain completely vegetative [see above and (170)]. This always occurred in Könitz's experiments, probably because the longest portion of any interrupted dark period was only about 1.5 hr. longer than the critical night. It is not valid to speculate about the quantity of photoreceptor pigment if saturating quantities of light are not determined. His experiment seems to be a further demonstration of the idea that plant response to F-phytochrome is a function of time during the inductive dark period.

Light quality during the light period.—Other results reported in the paper by Könitz (95) are most interesting. Flowering was not only inhibited by interruption of the inductive dark period with red light, but also by interruption of the intervening light periods with far-red light. This new phenomenon immediately suggests that flowering is inhibited both by F-phytochrome during the dark period, and by R-phytochrome during the light period. Bünning & Joerrens (22, 23) have since described an analogous situation in the diapause induction of an insect. Violet and blue wavelengths promote during the first half and inhibit during the second half of the daily cycle, while yellow or red light acts in an opposite fashion.

A further finding of Könitz (95) was that the inhibition brought about by far-red light applied in the middle of the light period could be reversed by red light. In a sense, this result makes interpretation of the initial observation much more difficult. As mentioned above, phytochrome responds to white light primarily as though it were a red source. This should be especially true of Könitz' fluorescent light, so rich in red and poor in far-red. If red will reverse the far-red effect during the light period, why doesn't the normal

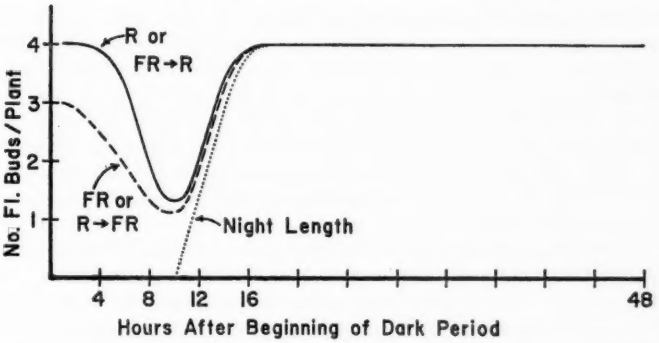

FIG. 1. The effect on flowering of *Pharbitis Nil.* by a light interruption at various times during a dark period of 16 hr. or more (199) (interruptions were not given at 9, 10, or 11 hr.). R = red; FR = far-red. For comparison, the response to various night lengths is also shown [data from (195, 196)].

white light of this light period reverse the effect as well and make the phenomenon impossible to observe? Furthermore, the Könitz results seem to contradict those of Meijer (125, 126) who found that far-red (or blue) was required during the light period for flowering of *Salvia occidentalis* Sw., a short-day plant. Stolwijk & Zeevaart (186) found the same thing for the long-day plant *Hyoscyamus*. Considerable literature (128, 129, 205) is accumulating on light quality effects during the light period. For example, it has been shown with *Salvia* that a light period of the proper mixture of wavelengths will allow a red light interruption of the dark period to promote instead of inhibit flowering (129). Subsequent research will probably be required for solution of these apparent paradoxes.

The pigment system in Pharbitis Nil.—Takimoto & Ikeda (193 to 200) and others (133) in Japan have been carrying out an intensive study of light interactions with the inductive dark period. Although Imamura (95) initially seemed to feel that *Pharbitis* was almost an exact Japanese counterpart of *Xanthium*, it has become abundantly clear that some of the responses of *Pharbitis* to light interruptions of the dark period are unprecedented by work with any other plant. The reader must be referred to the original papers for a complete discussion of the many details of response in this plant and the many points of controversy about these responses. A few high points will be presented here.

Two figures from one of the more recent papers (199) summarize many of the early results. These two figures have been generalized and combined into Figure 1. The curves show flowering response to red or far-red or either one followed by the other, applied at various times during a single inductive dark period. A curve showing response to different night-lengths (195, 196) has also been added. In the reported experiments, the dark period was 48 hr.,

but other papers (133, 197) seem to indicate that the shape of the curves would be essentially the same with any night-length exceeding 16 hr. It can be seen that both red and far-red inhibit flowering maximally when they are applied about 8 hr. after the beginning of the dark period, but far-red is also effective during the hours preceding this. Indeed, a dark period preceded by far-red (before time zero in Fig. 1) is less effective than one preceded by white light. This early far-red inhibition can be reversed by red (which in turn can be reversed by far-red, etc.), but red interruption effects at ca. 8 to 12 hr. are not reversed by far-red.

Borthwick, Nakayama & Hendricks (133a) have obtained essentially the same results at Beltsville, except that they observed, in the first part of a 16-hour dark period, a promotion with red that was reversed by far-red. Thus in their experiments red light (F-phytochrome) was promotive and reversible in the first part of the dark period, and inhibitory and not reversible in the middle of the dark period. They suggest that failure of far-red to reverse red is due to some short of quenching reaction of F-phytochrome with a substrate or an inhibitor. They point out that *Pharbitis* acts in a sense like a long-day plant during the first part of the dark period and a short-day plant near the middle of the dark period.

Another interpretation of why far-red fails to reverse red seems possible. Optimal flowering in *Pharbitis* may require a mixture or balance of the pigment forms near the middle of the dark period. This might be indicated by the fact that both red and far-red inhibited flowering, but neither inhibited it completely in the experiments of Takimoto & Ikeda (Fig. 1). Furthermore, they were able to obtain slight reversals of red with far-red or vice versa if the reversing light was applied at high intensities for short durations (10 sec.). Thus it seems possible that shifting the pigment system in either direction in the middle of the dark period might result in something incompatible with flowering. Only a very rapid, partial shift back to the dark condition would halt this process slightly. The Beltsville workers (133a) suggest however, that the far-red inhibition at the middle of the dark period is a result of absorption by R-phytochrome rather than F-phytochrome. The relatively high energies required would be due to low absorption by R-phytochrome at these wavelengths. Yet energies were about the same in the Japanese work (193, 200).

Takimoto & Ikeda (197) could find no evidence for a second high intensity light process in *Pharbitis*. Indeed, if plants were given a fairly short dark period (12 hr.), a light break, and another short dark period [4 hr., as in comparable experiments with *Xanthium* (116)], they flowered more than if they were simply returned to continuous light. This might be expected from the curves of Figure 1 since a light interruption at 12 hr. does not inhibit flowering as much as does returning the plants to full light at this time. If a balance in the pigment system is required at this time, one might imagine that a return to darkness would be more effective in allowing re-establishment of

this balance than would leaving plants in the light. Short dark periods following a light break at 16 hr. or later were without effect, also to be expected on the basis of Figure 1.

Many of the Japanese papers (133; 193 to 196; 199, 200) report that far-red (or low intensity light) preceding a long dark period (16 hr. or more) inhibits flowering [opposite to the finding of Borthwick *et al.* with *Xanthium* (13)]. They also state that far-red preceding short dark periods (12 hr. or less) promotes flowering. The critical dark period is shortened, but flowering is less than that of controls receiving a dark period equal to the time of far-red illumination plus the following dark period. The Japanese have stated that the first part of the dark period is relatively insensitive to light (e.g., 195) and that far-red or low intensity light preceding the dark period extends its length. But since this could only be true for 12-hour dark periods and not for 16-hour dark periods (far-red inhibits in this case), it seems to imply that the plant reads the mind of the experimenter and responds according to whether it is his intention to use a long or a short dark period! If far-red acts like darkness before a 12-hour dark period, why should it act differently before a 16-hour dark period? Actually, the promotion by far-red preceding short dark periods is only relative, as indicated above. It may be considered as an inhibition if the total time is considered rather than just the length of the following dark period. At any rate, the intricate arguments of the Japanese are illustrated, and we must again appeal to future research and clear thinking for clarification of the picture.

Flowering systems responding to a balance in phytochrome forms.—Several authors have shown that light containing a mixture of red and far-red (incandescent light) is most effective in lengthening the day and causing long-day plants to flower (51, 52, 53, 77, 153, 186, 189, 190). Takimoto (192) even reports that *Silene armeria* L. flowers best under continuous far-red. The Beltsville group (51, 52, 53, 153) explains this by pointing out that far-red causes stem elongation, a process common to all long-day plants used in the above studies. Indeed, in some plants stem elongation may lead to flowering. More commonly, flowering is a direct response to duration of light or darkness (a response to red light—F-phytochrome), but it may be aided by elongation in response to far-red (R-phytochrome). A balance in pigment forms may lead to optimal flowering. Only one non-Japanese paper known to the reviewer claims that incandescent light is more effective in a dark period interruption (long-day plant) than fluorescent light (62), which may indicate that maximum inhibition of a dark period depends upon a balance of pigment forms. If proven to be generally true, this suggestion might explain why a short duration of far-red will reverse a red interruption of a short-day plant, while a long duration of far-red is itself inhibitory (38, 50). Pure R-phytochrome maintained for a long period may be inhibitory, but returning plants to darkness may allow the proper balance to be established in time to reverse the red light effects.

What Is the Biochemistry of the Pigment's Action in Flowering?

In certain systems it has been possible to postulate an action of phytochrome in a specific step of a biosynthetic pathway [e.g., anthocyanin synthesis in apple skin (180, 181)], but its action in flowering is essentially unknown. Only a few general suggestions are available. For example, the inductive nature of the flowering process as contrasted to the non-inductive nature of many other photobiological responses (including anthocyanin synthesis) must have some bearing upon the problem. Downs (51) compares the inductive response to an on-off switch, while non-inductive processes compare to a modulating switch. In the "modulating" case it is possible to imagine that pigment in the proper excited form leads to an observed response that is directly related to amount of pigment. In the "on-off" case of flowering, however, the pigment system acts as a trigger, perhaps as a catalyst. It is as if some reaction leading to flowering is catalyzed by photoreceptor pigment in the proper form, or it is blocked or reversed by pigment in the improper form. In such a system, extent of the reaction may be highly dependent upon the concentration of reactants and products, the temperature, the time (e.g., night length), etc., as well as upon the catalytic pigment. The Beltsville workers (79, 81) have concluded that the biologically active form of the photoreceptor pigment is F-phytochrome. Downs (50) showed that far-red, to be effective, had to be applied within a short time after a red light interruption. Thus F-phytochrome seems to bring about some condition in *Xanthium* that is not reversed by reversing the pigment. In a later paragraph we will conclude that F-phytochrome is related to the flowering clock and that it will halt synthesis of flowering hormone. Direct biochemical research on the nature of phytochrome may be our main hope for a satisfactory solution.

TIMING IN THE FLOWERING PROCESS

Until the last decade the problem of biological time measurement has been either ignored by physiologists or relegated to a position of rather minor importance. Only since 1950–54 has the word "clock" been used in the physiological literature (154), yet the very term "photoperiodism" implies an ability of the plant to measure the duration of the photoperiod. It would probably be justifiable to assign the credit for the discovery of biological time measurement to Garner & Allard (64) who used the term "photoperiodism" in 1920. Zoologists (154) commonly assign this discovery to Beling (1) for his work on honey bees nearly a decade later.

Time measurement allows a plant to meet the requirements of natural selection and occupy a niche in space and time. A plant at a given latitude may time its growth and reproduction to the season if it is able to measure the length of day or night with accuracy. Withrow (218) has pointed out that an organism must be able to measure time with a precision on the order of 1 to 3 per cent if it is to measure seasonal time to one week, and 4 to 12 per

cent for an accuracy to one month. Since the rate of change in day length is greatest in the spring and fall and least in the summer and winter, the absolute precision required depends upon time of year. The unpredictable temperatures of natural environments require a high degree of temperature compensation if organisms are to measure time with the requisite accuracy. Response to the light intensities of twilight is also important. Because of the sensitivity to low light intensities (218), the photoresponses of the plant go from total darkness to saturating light intensities within a few minutes as the sun rises in the morning, and the extreme daytime fluctuations in light intensity brought about by clouds are "ignored" by the plant. Nevertheless, clouds during twilight may influence the measurement of day-length (66, 198).

In spite of the early lead by plant physiologists, most of the work done after the discovery of photoperiodism seemed to consider the problem of timing as of secondary interest, and research was concentrated upon other aspects of the flowering process. The problem was finally brought most directly to the attention of plant physiologists by Bünning (17, 19, 20) with his hypothesis relating flowering to his previous studies on endogenous rhythms. Persistent rhythms of leaf movement, flower opening and closing, and a great many other phenomena in both plants and animals, had been studied since at least the middle of the last century. These were shown by Bünning and other workers (19, 20, 154) to be, in many cases at least, endogenous in nature—i.e., the rhythms continued even though the plants were held under constant external conditions (within experimental limitations). Recently the term "circadian" has been applied to rhythms of this type that approximate 24 hr. in their period.

On the basis of leaf movement observations, Bünning (17, 19, 20) proposed that plants alternate every 24 hr. through a "photophil" and a "scotophil" phase. He applied the theory to flowering by proposing that light applied during the photophil phase promoted the flowering process and light during the scotophil phase was inhibitory to flowering. Light following a dark period set the phases (as could be shown with leaf movements). In long-day plants the rhythm was delayed so that an extended day-length or light interruption of the dark period fell during the photophil phase, while in short-day plants the rhythm was not delayed and a short-day allowed the onset of darkness to coincide with the scotophil phase.

The theory agreed fairly well with most of the facts of photoperiodism known by the middle of the 1940's, and some new evidences were marshaled as well. For example, experiments by Carr in 1952 (33) seemed to strongly support Bünning's hypothesis. *Kalanchoë* plants were given 72-hour dark periods. Light interruptions were applied at various times during these periods and produced peaks of inhibition spaced 24 hr. apart as well as 24-hour peaks of promotion. Carr concluded: "This experiment constitutes a critical test of the theory of Bünning which must therefore be regarded as

finally and decisively proved. It is desirable however that the experiment should be repeated with other short-day plants and with long-day plants, and it is hoped that this will be done in the near future."

Most plant physiologists, in the United States at least, were not so anxious to accept the theory. In retrospect, the principal reason for this reluctance appears to have been a feeling on the part of these workers that the theory of Bünning was something of an exercise in mysticism. Biological phenomena were to be explained in physiochemical terms. Thus unknown precursors, intermediates, and final flowering substances were postulated. Bünning, on the other hand, spoke only of phases that loved either the light or the darkness. The feeling was that even if Bünning's hypothesis were true, it had little meaning in terms of understanding biochemical mechanisms in flowering. Timing in the flowering process resulted from the rates of certain critical reactions, and the proper duty of researchers was to discover these reactions and their rate constants.

Experimental evidence and interpretations were accumulating that failed to agree with Bünning's theory. Carr (32) had already done experiments using 48-hour cycles (38 hr. darkness) and had found that light interruptions near the end or beginning of the dark period were most effective in the inhibition of flowering. Claes & Lang (44) had suggested that light interruptions may interact with the main light period if they are close to it. In extended cycle experiments with two long-day species, Hussey (84) concluded in 1954 "that so far as *Anagallis* and *Arabidopsis* are concerned, the persistent alternation of photophil and scotophil phases as postulated by Bünning cannot be the basis of the photoperiodic reaction." Experiments by Kribben (97) also failed to support Bünning, and Wareing (208) obtained data clearly opposed to the basic theory of Bünning. Light breaks near the beginning or the end of extended dark periods inhibited flowering whether they fell in the photophil or the scotophil phase, and the relationship of the initiation of the dark period to the beginning of the previous light period seemed unimportant. Bünning's theory failed in other ways as well (48). It should be noted that the explanations of many of these workers seem nearly as mystical as those of Bünning, but a concerted effort to postulate "substances" is usually evident, and reaction rates were frequently discussed (e.g., 79, 111, 133, 171). Many workers felt that Bünning's theory had collapsed and, unfortunately, there was also a feeling that since Bünning's theory had collapsed, no relationship existed between endogenous rhythms and timing in the flowering process.

Bünning then modified his theory (20) even to the extent of admitting that long- and short-day plants both exhibit the same scotophil or photophil characteristics at the same relative times of day. He recalled (20) that leaf movement rhythms would persist in some plants for only one or two 24-hour cycles under constant conditions, providing a possible explanation for the failure of extended cycle experiments, and that the cycle of some plants

is set by going from light to darkness rather than from darkness to light (17, 20). But Bünning still maintains that timing in flowering is related to endogenous rhythms, and indeed, he has recently reported on a similar situation in the diapause induction of an insect (22, 23).

In the past few years the problem has been reexamined. Hamner and his students (2, 73, 135, 136, 137) have carried out an extensive program of experimentation in which plants were given a series of day and night cycles of various total lengths. His results had been foreshadowed by an experiment of Wareing (207) in which it was shown that, using such cycles, if the nights are very long the days must be relatively shorter. In Hamner's much more detailed experiments it was evident that optimal flowering occurs when the light and dark periods total some multiple of approximately 24 hr. This does not prove Bünning's hypothesis, but it emphasizes the possible relationship between persistent circadian rhythms and the flowering process. The experiments of Könitz (95), in which it was shown that far-red inhibits when applied during the light period, also emphasize this possible relationship.

As may be evident from the above paragraphs, the fundamental question of timing in the flowering process is often buried in the details of a theory. Participants in an argument about endogenous rhythms often finally discover that no one knows what the issues are. Thus it might be well to summarize the problems that await solution.

The endogenous or exogenous nature of timing.—Most biologists believe that timing is endogenously controlled, in a manner somewhat analogous to a wind-up clock. Pittendrigh is a spokesman for this viewpoint (154). Brown (15, 16) almost singlehandedly defends the viewpoint that time measurement is exogenously controlled, as an electric clock, by some subtle fluctuation in the external environment (cosmic rays, for example). This is a basic aspect of the problem of biological timing, but it is probably of secondary importance to timing in the flowering process.

The pendulum or hourglass nature of the flowering clock.—This is the basic question raised by Bünning. Timing controlled by the rate of a simple chemical reaction would be analogous to an hourglass in that only one cycle could be timed, after which the reaction would have to be reinitiated (the hourglass turned over). An oscillating system such as a pendulum, however, must account for persistent circadian rhythms under constant environmental conditions. The Beltsville workers (11a, 79) are proponents of the reaction clock in flowering. They argue that the dark conversion time of F-phytochrome to R-phytochrome (half time of one or two hours) provides a simple solution to the problem of timing in the flowering process. Reduction of F-phytochrome to 3 or 4 per cent of its original level would take 8 to 12 hr. A pendulum theory would assume that there is a timing mechanism with a circadian period but that the pigment system is not this mechanism. Phytochrome would be only the linkage between the flowering clock and the light environment. For example, the reaction of F-phytochrome with its sub-

strates might depend upon the status (condition) of the clock. On the basis of the present marginal evidence, a decision between the two theories cannot be made. Two lines of reasoning seem to favor the pendulum theory, however,

First, the assumption that a light interruption of the dark period resets the flowering clock is implicit in the hourglass (pigment system) theory. (Such an assumption is not excluded by the pendulum theories.) Thus it was argued that the effectiveness of a light interruption in the first part of an inductive dark period depended upon the length of the remaining dark period (e.g., 171). Yet in plants treated with cobaltous ion (170), timing is slowed so that an interruption in the middle of a 16-hour dark period allows flowering, while a somewhat later interruption will not. Since the 8-hour interruption divides the dark period into periods less than the critical night, it is difficult to reconcile this with the idea that the clock is reset by a red light interruption of the dark period. Nevertheless, the clock that controls leaf movements is under the control of the phytochrome system and is reset by red light (122).

Second, it was shown (171) that nearly equal quantities of light were required to saturate the pigment when applied any time after the first three hours of an inductive dark period. This seems to indicate that pigment conversion is complete before the completion of time measurement, which agrees with older work (219) and with experiments of Borthwick *et al.* (13) (in which they were able to shorten the critical dark period only about two hours by converting the pigment with far-red light). When timing was slowed by the cobaltous ion the quantity of light required for saturation did not seem to be influenced (170). Of course, experiments that relate flowering to circadian rhythms (95, 137) also favor the oscillator model.

Third, a decay curve based on half times is nearly flat by the time only 3 to 4 per cent of the original quantity is left. Such a flat curve seems incompatible with the sharp critical dark periods that are observed. Could critical night be only the difference between 3 and 4 per cent of the original F-phytochrome?

One or more physiological clocks.—The parsimonious conclusion is in favor of a single master clock for the control of flowering and the various circadian rhythms, but the problem might be a very difficult one to resolve. Using models, it is quite possible to imagine a single master clock provided with a number of cams, each of which might be connected with the master clock through a different gear ratio so that all did not measure time according to the same period. Of course, it is well known that a number of superimposed rhythms may be observed in a given organism (e.g., 15). The important question here is: if the flowering clock is an oscillator, is it the same one that controls other plant rhythms?

The basic clock mechanism.—The nature of the flowering clock is of interest and importance regardless of its relationship to other rhythms. Bünning hypothesizes about a relaxation oscillator (19, 92), and Pittendrigh (154) has developed a fairly complex hypothetical system. Such mechanical

models, with their springs, pendulum wheels, and gears, are easy to devise, but chemical oscillators are less obvious. The restriction of temperature compensation makes this especially difficult (19, 105, 154) even though there may be some temperature dependence in plants (19, 214). Thus the special time-measuring phenomena of living organisms have no satisfactory biochemical models, let alone direct biochemical understanding. It seems quite likely that new approaches will be required to bridge the gap between physical models and biochemistry.

Two lines of research offer some promise. Bünning's group has established a possible relationship between biological timing and the nucleus. Bünning reported in 1957 (18, 19) that urethane (ethyl carbamate) and colchicine sometimes influence the exhibition of biological rhythms (leaf movements). Recently one of his students (92) extended the observations to a number of other compounds. Some were ineffective [or caused "transients" (154)], but a few such as KCN, $Co(NO_3)_2$, ethyl and methyl alcohol, and theobromine changed the period of the rhythm. Some of these, at least, are known to influence the nucleus (e.g., cell division). Furthermore, the volume of the nucleus varies with a circadian rhythm (25, 211). The second line of research is based upon the observation that cobaltous ion slows timing in the flowering process, and sulfhydryl protectors such as cysteine and glutathione will reverse this action (170). Since sulfhydryl groups are known to react with cobaltous ion in solution (54), they are not positively implicated in the flowering clock. Cobaltous ion does seem to be implicated, however, and sulfhydryl groups may be. Although both of these lines of evidence are marginal at best, any biochemical information is of interest.

SYNTHESIS OF FLOWERING HORMONE

Evidence for flowering hormones (promoters and inhibitors).—The evidences for existence of a flowering hormone have been reviewed in detail by other authors (e.g., 100, 114). Flowering may be brought about in a graft partner held under strictly non-inductive conditions of day-length (or temperature) by an induced graft partner of the same, or in some cases not the same, species. Grafts between long- and short-day plants have been successful in both directions, and day-neutral plants grafted to non-induced short-day plants will cause these to flower (100, 225). A few failures have been encountered (224, 225). An essentially sufficient evidence for a flowering stimulus is the response of the leaf to photoperiod conditions while it is the bud that differentiates to produce the floral organs. Assuming the absence of nervous or electrical influences, a chemical interchange is implied, and if this is not a nutrient or energy supplying substance, then by definition (147) a hormone is involved. Since flowering of photoperiodically sensitive plants is not induced by nutrients or energy supplying substances, we should make no apologies about use of the term "hormone." There are a number of possible complications, however. The hormone (chemical interaction) might be a flowering inhibitor produced in the buds and removed to the leaves that are

being induced, but the idea seems unreasonable, and there is no evidence to support it (114). Another possibility is that the leaf produces an inhibitor of flowering under non-inductive conditions, and production of this inhibitor is stopped by the act of induction (213). Guttridge has obtained very convincing evidence that this is the case in strawberry, a short-day plant (69, 70, 71). Perhaps his most impressive evidence is the induction of flowering by removal of leaves (71). This was considered as proof of the inhibitor concept by Lang & Melchers (103) working with the long-day plant *Hyoscyamus*, but defoliation does not lead to flowering in most short-day plants (114). Guttridge was unable to find evidence for a flower promoting substance in strawberry (70), but other workers compromise the ideas of inhibitors and promoters in flowering (78, 136, 173, 174). Schwabe (173, 174), using the short-day plants *Kalanchoë*, *Perilla*, and *Chenopodium*, obtained good evidence for inhibitors produced on long days that are interspersed between short inductive days (destruction of flowering hormone did not seem likely); yet, a positive-acting flowering hormone is also required in his scheme. He postulates the formation of an adaptive enzyme during successive inductive cycles and has recently added evidence to this concept (175). It is interesting to note that most research with *Xanthium* is best interpreted on the basis of a positive flowering hormone, but Bogorad & McIlrath (4) have shown that an inhibitor of lettuce seed germination is produced by *Xanthium* leaves on long days. The relationship of this obseravtion to flowering is not clear. Further impressive evidence for the existence of the flowering hormone is provided by experiments in which leaves of sensitive short-day plants are removed at various intervals following a single inductive dark period (88, 93, 165, 182). If plants are defoliated immediately following induction, they remain vegetative; a later defoliation allows flowering. Such experiments were designed primarily to study the translocation of this material, but their success argues in favor of a flower promoting substance and against the removal of a flower inhibitor by induction. Certainly species differences are to be expected, and it is likely that inhibitors play their most important role in certain long-day plants (114).

Time of hormone synthesis.—In short-day plants the flowering hormone is apparently synthesized during the part of the inductive dark period that follows the critical night—the time-measuring reactions of the dark period. This is evidenced by the observation that the rate of development of the floral bud following a single inductive dark period is a function of how much this dark period exceeds the critical night, although a "saturation" night length is usually reached (85, 111, 165). A red light interruption during the period of hormone synthesis acts much as would returning plants to the main light period (171). We may imply that F-phytochrome halts hormone synthesis.

Most workers in photoperiodism have considered the idea that some hormone is synthesized before the end of the critical night but that this amount is insufficient to cause flowering and may be destroyed in less than

24 hr. There is some evidence for this idea and it helps to explain certain observations. A destruction of flowering hormone seems indicated by the inhibitory effect of extra-long dark periods (72, 116, 171), and it has been suggested (111, 171) that applied auxin results in a destruction of flowering hormone. If synthetic auxins will result in a destruction, it seems reasonable to assume that the natural auxins of the leaf under normal conditions might also result in the destruction of a certain amount of flowering stimulus. Thus, on a regime of subcritical dark periods, some flowering hormone may be synthesized in the hours preceding the end of the critical night and then be destroyed by natural auxins before the end of the next critical night. If this is the case, any treatment to prevent this natural destruction, would, in essence, shorten the critical night. At least three such experiments with *Xanthium* have been reported. Plants grown under threshold conditions (dark periods slightly less than the critical or dim light of intensities slightly too high for flowering) were caused to form buds by application of anti-auxins (7, 111). *Xanthium* plants given low temperatures (143, 221; see also 201) or high pressures of carbon dioxide (31) immediately following a subcritical dark period were also induced to form buds. All of these experiments might be explained if plants normally produce a small amount of flowering hormone on subcritical dark periods, if this hormone is normally destroyed in the plant, and if the above mentioned treatments in some way prevent its destruction. Alternatively, one or more of these treatments might prevent F-phytochrome from halting hormone synthesis.

It is possible that time measurement in flowering may be a matter of phytochrome conversion followed by synthesis of small amounts of flowering hormone, amounts too small to last until the end of the next sub-inductive dark period. Results of experiments in which chemicals were applied to *Xanthium* plants at various times in relation to a single inductive dark period are in opposition to this idea. Cobaltous ion (see above) inhibits flowering only when it is applied before the end of the critical dark period. In addition, it extends the critical night and influences the kinetics of light interruption of the dark period. A number of compounds discussed below inhibit flowering when they are applied during the last part of the dark period but not when applied thereafter (46, 166, 172). Most of these have no effect on the critical night. Two (ethionine and picolinic acid) that lengthened critical night do not influence the kinetics of light interruption (46) and seem to influence critical night through a stoichiometric inhibition of flowering hormone synthesis. Thus two phases of the inductive dark period—time measurement and hormone synthesis—seem to be adequately separated (166).

Leaf sensitivity and temperature response.—Hormone synthesis appears to be most active in the most rapidly expanding leaves of *Xanthium* (93, 165). Since these leaves are probably most active in synthesizing metabolites in general, this is not surprising. Many plants are strongly influenced by temperature, but *Xanthium* does not appear to be highly temperature sensi-

tive (120, 164). Temperature has been studied as a variable in many experiments (e.g., 160, 206), but only recently have short periods of extreme temperatures been used to study specific steps in the flowering process. Schwemmle (176) found that flowering was inhibited most in long-day plants when the dark period was "interrupted" near the middle with low temperatures (5°C); and least when the interruption occurred near the ends of the dark period. Short-day plants reacted least when interrupted near the middle and most when near the ends. High temperature (45°C) interruptions act in an opposite way (133, 176).

Biochemistry.—Synthesis of the flowering hormone implies chemistry. In contrast to the question of timing which seemed to be neglected for some time, the question of synthesis of flowering hormone has been of primary concern since it was formulated in the 1930's (39, 74). Yet actual information is not much more extensive than that concerning timing. The problem has been the lack of a suitable assay for the flowering stimulus. The only measure of its presence so far is the flowering of a mature test plant, and this is complicated by a requirement that there be a phloem connection between the source of the flowering hormone and the bud that differentiates into a flower (89, 216). So far, the relatively few instances in which applied chemicals have caused flowering, rather than solving the problem, have tended to increase its complexity. Synthetic auxins and other chemicals applied to the pineapple plant caused it to flower [summarized in (10)], but no one felt that any one of these chemicals was itself the flowering hormone. Gibberellins will cause many species of long-day, cold-requiring plants to flower under non-inductive conditions (42, 43, 48); and promotions of flowering in short-day plants under certain inductive conditions have been reported (68, 108, 145, 146, 168). Chailakhyan (40) suggests that flowering may require two substances. Gibberellin is one of these and limits flowering in long-day plants; the other limits flowering in short-day plants. Yet most workers feel that the gibberellins do not constitute the elusive flowering substance (42, 101), although they do meet some of the originally established criteria. Extracts are obtained from higher plants (152), and such extracts may induce flowering (26, 104, 146). Indeed, plants induced to flower by the proper environmental conditions have sometimes yielded more of these gibberellin-like substances than comparable control plants (152). The only persistent claim for extraction[3] of the flowering substance (a lipid) comes from Roberts (158), but his methods have not been described in sufficient detail for duplication in other laboratories. Furthermore, it is very possible that his flowering substances are of the same category as the gibberbellins, or the auxins that promote flowering of pineapple—they may influence the flowering process without themselves being the flowering substance.

[3] In a manuscript accepted for publication by *Science*, R. Lincoln, D. Mayfield, and A. Cunningham describe experiments in which extracts are made at below zero temperatures from induced *Xanthium* leaves, incorporated into lanolin, and then smeared on vegetative plants, causing them to flower under strict long-day conditions. Unfortunately, vegetative control plants were not extracted (Jan. 1961).

Many early attempts were made to learn about the biochemistry of hormone synthesis by studying the changes in metabolites that accompany flowering (100). Changes in growth promoters have also been measured recently (75). In all cases there are so many changes that their meaning is not clear. Cause and effect cannot be separated. A sort of last resort has been the application of chemicals to whole plants.

Earlier reviews (10, 48, 111) have discussed the results obtained by the application of auxins. To summarize: the flowering of many plants is inhibited by applying auxins at relatively high concentrations, while flowering is promoted in a few plants, including pineapple and the litchi tree. Other plants have been promoted in flowering by application of auxins under special conditions [low temperatures (107), low light intensity (165), low concentrations to older leaves (222), and threshold day length (113)]. Since auxin inhibition may be overcome by simultaneous application of antiauxins (11), it is probable that the action of auxin in flowering is very similar to its action in other growth responses such as cell elongation. (Unpublished experiments of the author, however, indicate that reversal of auxin effects by anti-auxin is far more difficult to achieve than reversal of certain antimetabolites by the corresponding metabolites, as discussed below.) Evidences that the natural auxins of plant tissues influence flowering include not only promotion of flowering under threshhold conditions by antiauxins (7, 111), but promotion of flowering by ethylene (94), and maintaining plants in an inverted position[4] (60). All of these treatments were interpreted as lowering the natural auxin concentrations within the plant leaf. Clear cut inhibitions can be obtained only when quantities of auxin are applied that will cause quite serious side effects to the plant (48, 59, 171). The primary effect must not be upon flowering hormone synthesis because applied auxin inhibits flowering in short-day plants in the hours following the dark period as well as during the dark period (111, 171). Preliminary experiments indicate that auxin also does not influence the kinetics of hormone translocation (168).

When it became evident that auxin could not be implicated in the synthesis of flowering hormone, other chemicals were investigated. Liverman & Bonner (112) showed with short-day plants and Fredericq (61) with long-day plants that the requirement for high intensity light (72) could be satisfied by applying sucrose and other energy substrates to the plant. Lang (102) has shown in a preliminary way that reducing substances including glutathione, cysteine, and ascorbic acid will also fill this requirement. A requirement for sucrose or energy substrates implies that part of the dark process involves conversion of these substrates to energy—respiration. For a time respiratory energy exists in the form of reducing power, which may help explain Lang's observations. Respiration was also implicated by Nakayama,

[4] Wareing *et al.* describe an accelerating effect upon flowering by inverting the branches of fruit trees (210) or the Japanese larch (121). They do not speculate about endogenous auxin.

Fukamizu & Sei (134), who showed that cyanide, azide, fluoride, and arsenate will inhibit flowering of *Pharbitis* when applied during the second half of the dark period. 2,4-Dinitrophenol will also inhibit flowering of *Xanthium* when applied during the last half of the dark period (166). In the Japanese experiments malonic acid did not inhibit flowering, and they concluded that only the glycolitic pathways are essential for flowering and that the Krebs cycle phase of respiration was not. Results with dinitrophenol would indicate that ATP is essential.

Experiments with applied chemicals can be interpreted with some degree of confidence only if time of application data are available. A substance that inhibits flowering only in the early part of the dark period may affect only the timing mechanism, as with cobaltous ion. Compounds that influence flowering after the end of the inductive dark period may have little or nothing to do with synthesis of flowering hormone. Maleic hydrazide, 2,2-dichloropropionic acid (dalapon), and 2,4-dichlorophenoxyacetic acid (2,4-D) probably inhibit development of the floral bud, because they all inhibit flowering regardless of when they are applied (166).

Current research by the author with W. Collins and J. Bonner (46, 169, 172) concerns the effects of a number of antimetabolites upon flowering of *Xanthium*. Of twelve anti-amino acids that were tried (45), only one inhibited flowering. Thus protein synthesis may not be a part of flowering hormone synthesis. The one effective compound was ethionine, an inhibitor of methionine. Ethionine may inhibit donation of methyl groups from methionine. Quercetin, an inhibitor of rutin metabolism in animal biochemistry, and picolinic acid, which is known to inhibit thiamine metabolism in certain animal systems, inhibit flowering when they are applied during the period of hormone synthesis. The action of these two compounds in the flowering process is unknown.

A number of compounds that inhibit various phases of nucleic acid metabolism in certain systems have been tried with *Xanthium* (45, 172). Ineffective compounds include adenosine, 2-amino-4-methyl pyrimidine, barbituric acid, 5-hydroxyuridine, and 5-mercaptouracil. Compounds that inhibit flowering include 5-fluorouracil, benzimidazole, thiouracil, 2,6-diamino purine sulfate, and 8-chloroxanthine. As yet no reliable conclusions can be drawn from these lists since inhibitors of both RNA and DNA metabolism are found among the successful and among the unsuccessful groups. All of the above compounds have been tested in experiments designed to study the effect of a range of concentrations, the effective time of application, and effects on the critical night, but only 5-fluorouracil has been studied in any further detail (172). Of the above compounds that were successful in the inhibition of flowering, none influenced the length of the critical dark period in a clear cut manner (thiouracil and 2,6-diamino purine sulfate had slight effects), and all except one inhibited flowering only if applied before the end of the dark period (thiouracil also inhibited flowering when applied during the translocation period).

A test of whether an antimetabolite is acting in flowering in a manner

similar to that in other biochemical systems is to see if simultaneous application of the corresponding metabolite will reverse the effect upon flowering (169). Of the above compounds only ethionine and 5-fluorouracil have been adequately tested in this manner. Ethionine inhibition is completely reversed by simultaneous application of methionine (46), and 5-fluorouracil inhibition is reversed by orotic acid (172). Reversals of inhibitions by some of the other compounds have been accomplished in preliminary unpublished experiments. Attempts to reverse the effects of 5-fluorouracil with thymidine or uracil have been consistently and conclusively negative (172). Sucrose is also unable to affect the 5-fluorouracil inhibition (uracil is known to take part in sucrose metabolism). These experiments are being continued and the results so far seem to merit a more direct biochemical approach, which is also being undertaken.

A most interesting aspect of the work with 5-fluorouracil (172) was the finding that under certain conditions the inhibition caused by application of this compound was most complete when the material was applied only to the bud and not to the leaf. Subsequent (unpublished) experiments by Bonner and Zeevaart indicate that 5-fluorouracil may be translocated from the leaf to the bud but that it is never translocated from the bud to the leaf. Thus this compound must be effective in the bud during the period of flowering hormone synthesis.

Three possible conclusions come to mind. (*a*) Some process essential to flowering but sensitive to 5-fluorouracil is taking place in the bud during the last part of the inductive dark period. No evidence for such a process has ever been reported, but perhaps these experiments constitute sufficient evidence to warrant a detailed search. (*b*) Some material other than the flowering stimulus (which is typically translocated following the dark period) is translocated from the leaf during the dark period. Action of this material in the bud is inhibited by 5-fluorouracil. This explanation postulates two flowering hormones (9, 172). (*c*) The effective time of application of 5-fluorouracil is a coincidence caused by a delay (slow reaction rates) in its inhibition of processes that normally occur in the bud. Thus it must be applied a day or two before the flowering stimulus reaches the bud. Such a delayed reaction seems unlikely.

To summarize: experiments with anti-purines and pyrimidines have implicated nucleic acid metabolism in the flowering process, and results of the time of application experiments have tied this effect to the period of flowering hormone synthesis. The results with 5-fluorouracil have located a response in the bud rather than the leaf, the known site of hormone synthesis. There is every reason to believe that perusal of these anomalies, especially by direct biochemical research, will lead to considerable further clarification of the flowering mechanism; but at present the perplexities are much more obvious than the explanations. At least localization in the bud of the nucleic acid effects saves us from the necessity of immediately postulating translocatable nucleic acids.

An exciting aspect of the antimetabolite work is that when the inhibition

due to an antimetabolite can be reversed by its corresponding metabolite, this is evidence for participation of the metabolite in the flowering process. Attempts have been made in the past (98, 179, 183) to label the flowering stimulus by allowing the plant to photosynthesize in the presence of radioactive carbon dioxide. These have been uniformly unsuccessful because labeled carbon dioxide ends up in nearly all of the compounds of the plant. If specific precursors are identified, however, labeling them and introducing them into the plant at the proper time might produce more acceptable results (9).

THE SECOND HIGH INTENSITY LIGHT PROCESS

In relatively recent years the work of Lockhart & Hamner (115, 116) has renewed the discussion of the effects of light conditions following the inductive dark period. Early work (72, 124) indicated that low intensity light either preceding or following the inductive dark period reduces subsequent flowering. Lockhart & Hamner studied this phenomenon in detail by giving *Xanthium* plants an inductive dark period, a relatively short light break, and then a short period of darkness (usually four hours). This post-inductive dark period inhibited flowering. Unfortunately, they confused the issue somewhat by assuming that applied auxin (IAA) had the same effect as the post-inductive dark period, and thus in most of their experiments auxin was applied before this short dark period. They concluded that the period of hormone synthesis during the last part of the inductive dark period produced only a precursor of a flowering hormone which then had to be stabilized by high intensity light applied to the leaf. Their alternative explanation seems more logical to the reviewer: some leaves (depending upon age or as yet unknown environmental conditions) may cause the destruction of flowering hormone in darkness, and high intensity light changes the condition of such leaves so that they are no longer capable of destroying flowering hormone. The data fit either explanation almost equally well, but the second one does not require a second synthesis step. It is not known why some leaves have this ability while others do not, but a post-inductive dark period is not always effective (36, 72, 115, 116, 164).

Some problems were evident at the time of Lockhart & Hamner's experiments, and others have become apparent since that time. To begin with, it was known that even *Xanthium* will flower if it is left in total darkness for about a week (plants die after an extended period) (72). Considerable data have been accumulated to indicate that many plants will flower under conditions of total darkness. These include short-day plants (72, 203), long-day plants (58, 106, 202), and day neutral plants (106, 188). Both etiolated and green plants seem to respond in this way although some do not (174). If only *Xanthium* were being considered, one might say that these results certainly eliminate the rigorous requirement for a second high intensity light process. Since so many plants flower in extended darkness, however, it is tempting to imagine that this is a special situation, one that may bypass in certain re-

spects the normal steps in the flowering process. If this were the case, high intensity light might still be required under "normal" conditions. As discussed above, however, a second high intensity light process cannot be demonstrated in *Pharbitis*.

Carr (36), using *Xanthium*, was able to overcome the requirement for a second high intensity light period by treating the plants with sucrose. Previous attempts at this had failed (111, 164). Carr concluded that the second high intensity light process was not a stabilization of flowering hormone precursor in the leaf but simply an effect upon translocation. Photosynthate was required to move the flowering hormone out of the leaf either by mass flow or by providing energy for an active movement. There must also be a destruction of flowering hormone in the leaf, otherwise it should simply be moved out at the close of a post-inductive dark period when plants are finally returned to high intensity light conditions; and post-inductive inhibition could not be observed. Metabolism of sucrose may change the leaves so that they are incapable of hormone destruction, or the hormone may be moved out of the leaf before any destruction can occur [the second possibility seems less likely since hormone is usually not moved out so soon (116)]. If the destruction in the post-inductive dark period is because of relatively high auxin levels in the leaf, as suggested by Lockhart & Hamner (115, 116), then sucrose metabolism might be responsible for the lowering of these auxin levels. It might also result in stabilization of a hormone precursor. At any rate, Carr's experiments seem to indicate that the second high intensity light process is related to photosynthesis.

THE INDUCED STATE

So far the discussion has considered only the act of induction (165): the events occurring within the plant that result in the formation and translocation out of the leaf of a flowering stimulus. Many plants are capable of attaining the induced state (8, 48)—that is, flowers will continue to develop even though the plant is returned from the inductive day-length conditions to a non-inductive photoperiod regime. Plants that require only a single inductive cycle are the most extreme examples of this category. Other authors have used different terms in the discussion of these concepts (67, 117).

Translocation of the flowering stimulus from the leaf.—Initial translocation (see discussion of defoliation experiments above) must be a part of the act of induction, but certain aspects of the subsequent movement of hormone within the plant seem to be characteristic of the induced state. Therefore, translocation will be discussed under that title. It was shown almost two decades ago by Withrow & Withrow (216) that the flowering stimulus would move only through living tissue in *Xanthium* and soy bean. It will move in either direction. Recently Imamura & Takimoto (89) have demonstrated the same things in *Pharbitis*. Selim (178) claims xylem movement in *Perilla*, but this is disputed by Zeevaart (225). Dissipation of the hormone in the

stem of *Pharbitis* has been studied (90) and the rate of movement was found to be 2.6 to 3.8 mm. per hour (86, 87). These rates are in agreement with rough estimates made by less ingenious methods (100) and indicate that movement of the flowering hormone is one or two orders of magnitude slower than movement of other organic materials through the phloem elements. This observation might be a clue to a means of attack of the flowering stimulus in isolation as suggested by Bonner (9), and it also may indicate a high molecular weight for the flowering hormone.

Many authors (76, 100, 109, 220) have found that transmission of the flowering stimulus may be inhibited by leaves maintained on a non-inductive day length. Various explanations of these results have been forthcoming: the leaves may "absorb" the flowering hormone as it goes by; leaves on the non-inductive day lengths may produce inhibitors of flowering (see above); or such leaves may otherwise prevent the hormone from reaching the bud (41).

Some recent studies by Carr (37) are most stimulating. On the basis of leaf graft experiments and *in vitro* culture of induced and non-induced apical buds, he concluded among other things that the flowering stimulus is initially moved completely out of the leaf, leaving the leaf devoid of this substance. Then after a few days the hormone reappears, after which the leaf is truly in the induced state. It is well known (100) that young *Xanthium* leaves that were not subjected to the inductive cycle but grew out after the plants had been induced, also are converted to the induced state. Lona (117) and Zeevaart (225) have shown that young *Perilla* leaves are not so converted, although the leaf initially subjected to the inductive dark period is in the induced state from the act of induction until it dies.

Necessity for active buds.—Although this topic was reviewed in detail by Doorenbos & Wellensiek (48), the subject should be briefly outlined again for the sake of completeness. In early experiments Lona (118) claimed that leaves that had been detached from the plant were still capable of the act of induction. Carr (34) was unable to induce detached leaves, but Lona's group again succeeded (3), and Zeevaart induced detached *Perilla* and *Xanthium* leaves and disbudded plants by giving at least ten inductive cycles (223, 225). Nevertheless, in experiments using disbudded plants and only one or a few inductive cycles (35, 110), it was shown that plants disbudded at the right time were incapable of transmitting the flowering stimulus when grafted to plants having buds. "Inactive" buds were used (110, 165) to obtain essentially the same results without the necessity of grafting to a receptor plant. It was shown by experiments of this type that if active buds were removed just previous to or any time following the act of induction, inactive buds would be vegetative when they became active. If they were to receive the flowering stimulus and become reproductive, they would have to become active before the stimulus was "dissipated" from the plant (about four to six days). It was shown that the requirement for active buds could be replaced by auxin (165), or by young leaves (110) recently shown to produce auxin [or something that maintains axillary buds in a dormant condition

(5)]. It was concluded that flowering stimulus was "preserved" by the presence of auxin until inactive buds can become active (165). Gibberellin may help young tissues preserve the stimulus (108). Carr (36) and Zeevaart (225) suggested that the effect of auxin was merely to make the buds become active sooner, before the hormone had a chance to become "dissipated." It is well known, however, that auxin usually inhibits the growth of axillary buds rather than promoting it, and, indeed, unpublished observations by the author during the original experiments bore out this idea.

It was observed (165) that when active floral buds were removed up to at least seven days after induction, inactive buds were vegetative when they became active. Thus it is possible to cause a plant to revert from the flowering to the vegetative condition by the removal of the flowering buds. Young leaves tend to prevent this reversion (110). Recently, this phenomenon has been reinvestigated by Lam & Leopold (99).

THE ACTION OF FLOWERING HORMONE

Probably the most fundamental question in biology concerns the nature of morphogenesis. It is a phenomenon common to all living things (even the virus particle is organized after the synthesis of its constituents) and is responsible for the myriad forms of life that surround us. Beyond the idea that it may be controlled by certain chemical entities, we know essentially nothing about the process. Indeed, the very problem of how a chemical can control the formation of a three dimensional structure seems to be beyond the approach of our present research methods. The flowering hormone is apparently such a chemical, and flowering should be an ideal situation for study of morphogenesis. In sensitive plants such as *Xanthium* the time of differentiation can be determined and controlled within a matter of hours. Morphogenesis encompasses an even more perplexing problem than the one mentioned above: how do the genes control specific species characteristics of a morphological nature? In flowering, the releasing trigger that allows the species-characteristic floral genes to act seems to be the arrival of the flowering hormone at the meristematic site. Thus virtually all phases of morphogenesis could be studied with the flowering process as a basic frame of reference.

Studies in this field, however, have been extremely limited. Although there has been a great deal of general descriptive work relating to the development of floral organs, etc. (55), work with photoperiodically sensitive species directed towards understanding the cellular and tissue changes that take place at the apical meristems during floral differentiation have been very few in number. F. L. Naylor (142) published a description of the surface changes that take place in developing *Xanthium* inflorescences, but no attention was paid to changes at the cellular level. Millington & Fisk (131) published a paper on the vegetative characteristics of the *Xanthium* apical meristem, but a promised paper on the floral apex has never appeared. Popham & Chan (155) described the developing inflorescence of *Chry-*

santhemum morifolium, a short-day plant. Thein (204) studied the flowering shoot of *Xanthium*, including some chemical changes. Sachs and co-workers (162, 163) have studied cell divisions in the stems of bolting long-day plants, especially as gibberellins influence the process, but this is not a study of floral differentiation itself although it does approach a truly experimental study of the problem. Wetmore and co-workers (215) have published a recent description of the cellular changes taking place in the differentiating apical meristem. Little information exists on the biochemical changes that take place in the differentiating meristem. Some pioneer work has been done on the biohistochemistry of vegetative apices [reviewed by Wetmore *et al.* (215)], but intensive histochemical work on differentiating apices of photoperiodically sensitive plants remains to be done.

The work of Wetmore *et al.* (215) was admittedly preliminary, and the experiments could easily be improved. They were overcomplicated by use of various numbers of inductive cycles; samples were taken only in duplicate and only every other day, while the initial changes that occur must take place within a matter of hours. Nevertheless, their description of what happens is the best available at this time. Their observations were summarized in five points. (*a*) The cells just below the "central zone" in the apical meristem were the first to become active in the differentiating bud. This activity consisted of cell divisions and some enlargement and was found in all species studied by this group, as well as in the literature cited by them. This might be the most important finding of the entire study. Perhaps research efforts should be directed towards biochemical understanding of this phenomenon. (*b*) Activity then spread to the other cells, including those in the central zone. Cells in this zone become smaller by dividing, and eventually there is a layer of meristematic cells two or three cells deep covering the entire bud. The bud at this stage is an easily recognizable floral primordium and has passed far beyond the initial changeover from vegetative to reproductive state. (*c*) Enlargement of the pith rib meristem cells causes swelling of the bud. (*d*) The layer of meristematic cells covering the surface forms the bracts, flower parts, etc. (*e*) Extension of the axis stops, and apical dominance is lost.

To the present reviewer one of the more unfortunate aspects of work in this field concerns the time wasted in quite philosophical discussions on the controversy of the meristem *d'attente*. The French school of morphologists under Grégoire (65) and Buvat (30) suggested that the central zone of the apical meristem consisted of inactive cells simply lifted into space as the stem grew, and that such vegetative stem growth originated from division of cells below this group. At the time of differentiation of the floral bud these cells, the meristem *d'attente* (the meristem in waiting), became active and developed into the flower. If this theory were true, it would, of course, have implications for the physiologist—only the cells of the meristem *d'attente* must respond to flowering hormone. Experiments with photoperiodically sensitive plants should settle the controversy once and for all. Indeed, the

five points of Wetmore *et al.* based on such actual observation fail to support the theory, and the French school has retreated considerably (47).

At any rate crucial problems in the physiology and morphogenesis of flowering still await the efforts of future research. The basic experimental procedures are quite obvious. They need only to be done by someone trained in the proper techniques. Refinements of the obvious techniques have been suggested by Green and Erickson at the University of Pennsylvania (personal communication). These include the use of identical plants, so that identification markers such as axillary bud primordia might be chosen. Such markers would be very valuable for interpreting the changes in anatomy that accompany floral differentiation. Identical plants could probably be obtained, even with *Xanthium*. Plants might be chosen very carefully according to leaf size, height, age, and direction of phyllotaxy, and grown under carefully controlled conditions. Any work in this field should provide a groundwork for solving some of the problems of morphogenesis. And in view of the suggestions made above in the section on synthesis of the flowering hormone, a most exciting subject for histochemical study might be the DNA and RNA status within the various cells of the differentiating apical meristem.

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PHYSIOLOGY OF MITOSIS AND MEIOSIS[1,2]

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Introduction

Mitosis is usually defined as the division of a nucleus during which chromosomes become visible, and separate in such a fashion that two genetically equivalent daughter nuclei are produced. Meiosis is a modified mitosis or sequence of two mitoses that occurs only at one stage during the life cycle. Both types of division are frequently correlated with a cytoplasmic division, cytokinesis, and various other cellular changes so that a discussion of mitosis usually involves the whole cycle of cell division. During the last few decades evidence has accumulated which demonstrates that the events during the cycle between the visible division stages, i.e., during interphase, are in many respects more significant for understanding mitosis than the visible manifestations of prophase, metaphase, anaphase, and telophase.

If we adhere to the definition of mitosis as given, then there are many cells that divide without mitosis. To relegate all of these cases to the category of amitosis would be a mistake since this term often implies an unorganized division and, as such, only occurs in pathological conditions or in nuclei with a limited genetic future. As a result, the term mitosis may evolve to mean any division in which genetic evidence shows that there is a regular distribution of genetic material. Likewise, the term chromosome is being used to refer to a linear arrangement of genetic units whether or not it ever appears as a visible structure. For example, the bacteriophage chromosome and the chromosome of *Escherichia coli* are useful concepts in discussing their genetics. Although the chromosome of *E. coli* apparently never condenses (45), it duplicates and separates in a regular fashion as demonstrated by genetic evidence. Indeed, unless an organism has more than one chromosome, mitosis, i.e., chromosome condensation and spindle formation, would not appear to be necessary. Perhaps mitosis only became necessary in evolution when the organism acquired more than one chromosome or genetic linkage group.

In this review no attempt will be made to discuss mitosis and meiosis in separate categories. Although many obvious differences exist between the two types of divisions, the evidence to date reveals no striking physiological differences. The principal difference, of course, is in the length of the prophase of the first meiotic division in which pairing of chromosomes and formation of chiasmata occur. These events, correlated with a unitary behavior of centromeres at the first division, result in the reduction and segregation of chromosomes typical of meiosis. The timing and sequence of DNA replica-

[1] The survey of literature pertaining to this review was concluded in August 1960.

[2] The following abbreviation will be used: IAA (indoleacetic acid).

tion, and the synthesis of nuclear proteins in relation to the premeiotic interphase and prophase of the first division are not significantly different from other divisions (85). A number of reports have indicated differences or unusual phenomena in the behavior of nucleic acids, but closer examination has revealed these reports to be in error. Likewise, Darlington (15) has consistently maintained that the meiotic nucleus enters prophase precociously compared to nuclei in regular divisions. Again there is little or no biochemical evidence for this view; the DNA (deoxyribonucleic acid) is usually replicated in premeiotic interphase as in any other division. One unusual behavior of the chromosomes is the failure of their two chromatids to become visibly separate early in prophase, as they do in most mitoses. The factors determining the separation of chromatids are unknown, however, and the timing and degree of separation are variable when different tissues are compared at mitosis. The variations do not appear to be conditioned by the time of reproduction of the major chromosome components.

The Division Cycle

Interphase.—The duplication of the chromosomes occurs in interphase. The first measurements of the amount of DNA per nucleus that were made in tissues where divisions were occurring indicated that the synthesis of DNA occupied only a portion of the interphase stage, probably the middle part (84). Later, Howard & Pelc (37) used autoradiography and P^{32} to chart the events of the cell cycle as related to the incorporation of P^{32} into DNA. With the introduction of tritium-labeled thymidine for autoradiographic studies of DNA synthesis (94), a better method became available for studying the kinetics of the cell cycle. Friedkin, Tilson & Roberts (23) had shown by the use of C^{14}-thymidine that this nucleoside was a highly selective label for DNA, but the production of H^3-thymidine of high specific activity added a convenient and less expensive tool for studies of DNA replication at the cellular level. Its use in studying the events of the cell division cycle is illustrated by several papers (39, 63, 67, 90, 104).

The technique is well suited for an asynchronous population of cells with a uniform cycle. The cells are exposed to the thymidine-H^3 for a few minutes, then washed and changed to a medium with an excess of non-radioactive thymidine. Those cells in the process of DNA replication will become labeled. If the labeling time is short compared to the whole cycle, the ratio of labeled to unlabeled cells is proportional to the synthesis time divided by the time for the remainder of the cycle. The time required for labeled cells to reach division is a measure of the post-DNA synthesis period of interphase. If the division stages are of sufficient duration, a rough measure of their length can also be obtained from the interval required for labeled cells to arrive at the given stage less the post-DNA synthesis period. The pre-DNA synthesis period is obtained by the difference between the length of the whole cycle and the sum of the other time intervals. The whole cycle is measured by the interval between the arrival of a population of labeled cells at the first division and their arrival at a second division. In practice, populations of cells

sometimes vary enough in the length of the cycle and the length of the different phases that such data may not reveal discrete time intervals. The length of the whole cycle may be obscured because a well defined second peak of labeled division figures fails to appear.

Only one report of the cycle in a plant tissue has utilized this method. Wimber (104) studied the cycle in the root tip of *Tradescantia*. The second peak was not discrete enough for determining the length of the cycle; therefore, he attempted to determine the length of the interval of DNA replication by other means. He plotted the frequency of division figures against time on the abscissa of a graph, as in all such studies, but then used the duration of the first peak, i.e., the time required for the population of labeled cells to pass through mitosis, as a measure of the time of synthesis. He determined the slope of the ascending and descending part of the curve and then took as the synthesis time the interval between the 50 per cent points on the two slopes. From this interval (10.8 hr.) he calculated that the whole cycle was about 20 hr. at 25°C., the post-DNA synthesis period 2.7 hr., mitotic stages 2.5 hr., and the pre-DNA synthesis period 4 hr.

In a study of a population of connective tissue cells isolated from an embryo of Chinese hamster, Taylor (89) used the pulse labeling technique and was able to obtain a distinct second peak in the frequency curve of labeled mitoses. Using the interval between the two peaks, the cycle was estimated as 14 hr. with DNA synthesis occurring in about 6 hr. The post-DNA synthesis period to metaphase was 2 to 3 hr. The division stages were later estimated to require about 30 min. from mid-prophase to mid-telophase (90). Van't Hof, Wilson & Colon (95) have recently reported some success in measuring the mitotic cycle in roots of *Pisum* by the use of colchicine. They induced polyploidy in a fraction of the cell population by a 30-minute treatment of the roots with the drug. A part of the population of cells was thus marked and synchronized in the division cycle. The roots were removed from the colchicine solution, allowed to recover, and at intervals the frequency of polyploid cells at mitosis was determined in a sample of the roots. When the frequency of polyploid mitotic figures was plotted against time, three peaks in the curve appeared at intervals of about 12 hr. Although the third peak was broader than the first two and therefore indicated increasing asynchrony, the intervals between the appearance of the polyploid cells at division should be a valid measure of their cycle of division. For measurements of similar parameters, Hejnowicz (35) used a technique of sequential photographic records of root growth, plus determination of cell density along the root and frequency of mitotic stages at successive distance intervals (0.05 mm.) from the tip. From these data he calculated that the rate of cell formation per cell was nearly constant throughout the meristem (0.6 to 0.7 mm.) in these roots, possibly excluding a small relatively inactive region near the tip. Clowes (14) had confirmed the existence of such a region just back of the tip in another type of root by means of radioisotopes and autoradiography. Hejnowicz then calculated the duration of the whole cycle (average time between the two successive cell divisions) on the basis that it is given

by the reciprocal of the rate of cell formation per cell. The time calculated varied between 20 and 26 hr. for roots grown at 18°C.

All three of the methods mentioned above have possible defects and a comparison of results from all three methods on the same material would be interesting. For example, if too much isotope is incorporated in the labeling method, a mitotic delay could occur that would affect the determination of the cycle. Likewise, the tetraploid cells would perhaps be expected to divide more slowly than the regular diploid ones. The determination of new cell formation per cell is laborious and neither it nor the colchicine method allows the timing of other metabolic events in interphase in the way that the labeling method does.

In most rapidly dividing tissues the synthesis of DNA occurs in the middle of interphase and occupies about one-third to one-half of the cell cycle. Autoradiographic evidence has been presented which indicates that the synthesis of proteins of the nucleus coincides with DNA synthesis in root tip cells (38) and in cells at premeiotic interphase (88, 92). Later, cytophotometric measurements of basic proteins in individual nuclei indicated that the synthesis of these keeps pace with DNA replication (1, 4). Rasch & Woodard (68) measured the amount of basic proteins by cytophotometry in a variety of tissues of *Tradescantia*, lily, corn, onion, and broad bean. The fast-green staining technique introduced by Alfert & Geschwind (2) and the extraction of methanol-fixed tissues with 0.01 *N* HCl after removal of DNA were used to characterize and measure the basic proteins (histones). The amounts of nuclear histone fell into approximate multiples of the gametic (egg or sperm) quantity except in meristematic tissues where DNA replication was occurring. Here intermediate amounts of histone occurred in nuclei that were presumably in the process of chromosome duplication.

Amounts of histones per nucleus in root tip nuclei of *Tradescantia*, when plotted against interphase time, showed that the amount of histones was doubled during the middle third of interphase. The basic proteins of sperm nuclei of higher plants were found to be of the histone rather than protamine type; the latter being found in trout and some other animal sperm cells (68). Recently, by autoradiographic studies with tritium-labeled arginine, De (20) has confirmed the finding that DNA replication and basic protein (histone) synthesis are simultaneous in root cells. In addition, he has shown by the same technique that the synthesis of histones coincides with DNA replication in *Tradescantia* microsporocytes. Synthesis of both components is completed by early leptotene, one or more days before pairing of meiotic chromosomes can be detected.

By far the largest fraction of nuclear proteins in growing cells are the various non-basic proteins. The histones may represent nearly one-fourth the total in roots, but in many animal cells the ratio is smaller. Probably some proteins are produced continuously in the cytoplasm, and during interphase and prophase in nuclei. Support for this idea comes from the observation that uptake of labeled amino acids is continuous at all stages (74a, 90), and from the continuous increase in nuclear volume and non-basic proteins shown

by cytophotometric measurements of fast-green bound by staining at pH 2.0 (105). These latter measurements also indicated a decrease in total proteins during the division stages. On the other hand, Grun (30) used cytophotometric techniques based on ultraviolet light absorption and reported that *Tradescantia* root cells did not show a protein increase during interphase. He assumed the increase must occur during division stages; however, this conclusion is contrary to all other reports and is probably in error.

De (20) reports that during DNA replication in onion roots, accumulation of non-basic proteins by the nucleus is very slow compared to other periods in interphase. In partial agreement with this, cytophotometric measurements by Woodard *et al.* (105) indicates that the most rapid accumulation of non-basic proteins occurs in late interphase and perhaps in early prophase.

Taylor (85) has summarized the evidence that the chromosomes are duplicated, i.e. become double as indicated by radiation breakage, simultaneously with the synthesis of their two major components, DNA and basic proteins. In meiosis the chromosomes show their functional doubleness prior to prophase by the types of rearrangements that are produced following irradiation (56, 86, 88). As in other division cycles, the transition to doubleness occurs simultaneously with DNA replication and synthesis of the major portion of the nuclear proteins.

Although DNA replication frequently occurs during middle interphase, variations have been found. Replication may occur at early or late interphase in microspores and, as mentioned above, synthesis extends into early leptotene of meiotic prophase in *Tradescantia* microsporocytes. However, in the microsporocytes of *Lilium*, synthesis is completed well before the typical leptotene chromosomes become visible (88, 93). Replication of DNA may sometimes occupy the whole interphase in embryonic tissues, but synthesis has not been observed during the stages when chromosomes are condensed at prophase or other mitotic stages.

Since chromosome reproduction frequently appears to be completed in the middle third of the cell division cycle, the question of what occurs during the remainder of interphase may be asked. In a few instances the synthesis of RNA, which seems restricted to the nucleus in many kinds of cells (90), does not coincide with the period of DNA replication. In the microspores of *Tulbaghia* the two types of nucleic acids are not produced simultaneously (86) and some evidence was presented to indicate that root tip cells of *Tradescantia* were similar in this respect (74a). This is not a general rule, however, and in many cells the incorporation of isotopes into both types of nucleic acids can be observed simultaneously (90, 106, 107). Even if DNA serves as the template for all RNA synthesis, the two processes could occur simultaneously in nuclei, for it is now clear that all of the DNA is not replicating at one time. A genetically controlled sequence is indicated in several cell types (24, 51, 87, 89).

Whether there is a metabolic turnover of RNA in rapidly dividing cells is still an unsettled question and until it can be satisfactorily answered, the

related problem of the site of RNA synthesis is difficult if not impossible to solve. In 1954 Hershey (36) presented evidence for the conservation of both DNA and RNA during bacterial growth. Later, Siminovitch & Graham (73) showed that there was no loss of P^{32} incorporated into the RNA of rapidly growing mammalian cells in culture. These experiments show a conservation of nucleotides, but do not rule out the breakdown and efficient re-utilization of the nucleotides. Davern (19) has shown, however, that RNA of *Escherichia coli* does not undergo a detectable turnover. He labeled RNA with the stable isotopes C^{13} and N^{15}, and then transferred the bacteria to a medium with nutrients containing the light isotopes, C^{12} and N^{14}. Samples of RNA taken from the culture at intervals and separated by gradient-density centrifugation did not reveal RNA of intermediate densities as would be expected if the heavy polynucleotides were being broken down and re-utilized. The heavy molecules persisted intact during the subsequent growth and divisions of the cells.

Various experiments indicate that a turnover or breakdown of RNA may occur in some non-growing cells. For example, Watts & Harris (100) found that the macrophage from the rabbit, when placed in culture containing unlabeled adenosine, in 2 hr. lost about one-half of the C^{14}-adenine introduced in the RNA during the previous 4 hr. Slight loss occurred when inosine was added and no loss if inosine-5-phosphate was used for dilution. Later experiments by Harris (34) indicated a turnover of nuclear RNA in a rapidly growing connective-tissue cell in culture. A turnover of cytoplasmic RNA was not detected in the cells. Harris concluded that the nuclear RNA that disappeared did not appear in the cytoplasm in a stable form because the nucleus continued to lose labeled RNA after an increase in the cytoplasm had already occurred. His argument concerning this point is not entirely convincing, however. His data show some gain in cytoplasmic label during the period of loss. Considering the quantity of labeled RNA in the nucleus as compared to the cytoplasm, a transfer during the latter part of his experimental period might not have caused a significant change in the grains produced in autoradiographic film. A small loss of cytoplasmic label likewise would have compensated for the possible transfer. On the whole, however, his data are at least suggestive of a rapid turnover of a part of the nuclear RNA even in rapidly dividing cells. In addition, the autoradiographic studies of McMaster-Kaye (54) indicate a rapid turnover of the nucleolar RNA in the salivary gland cells of *Drosophila*. In such a situation the transfer of nuclear RNA to the cytoplasm is difficult to prove or disprove. However, there is considerable evidence that most if not all RNA is made in the nucleus. There are, for example, the experiments demonstrating that enucleated cells or cell fragments do not produce RNA; see Prescott (66) for a review.

In a recent paper Naori, Naori & Brachet (59) have re-evaluated the situation in enucleated fragments of *Acetabularia*. Work from this laboratory had indicated originally that a net increase of RNA occurred in enucleate fragments of this alga. Later measurements by Richter (69, 70) showed no net increase in the RNA of enucleate fragments of *Acetabularia*. Likewise

these measurements reported by Naori *et al.* (59) show no net increase, but carbon-14 was incorporated into the RNA when presented as orotic acid-C^{14} or adenine-C^{14}. About 80 per cent of the cytoplasmic RNA in this alga is bound to a large particulate fraction in homogenates that includes the chloroplasts. It was this fraction that became labeled in the enucleate fragments; the microsomal and soluble RNA remained unlabeled. In fragments with nuclei all fractions became labeled although the amount of radioactivity in the microsomal and soluble RNA was small compared to that in the large particle fraction. Although no net increase in RNA occurs in the absence of the nucleus, the authors (59) concluded that the incorporation of C^{14} into "chloroplastic" RNA indicated synthesis independent of the nucleus. Carbon dioxide-C^{14} was incorporated into purine bases both in nucleated and in enucleated pieces at about the same rate. The failure of a net increase of RNA to occur in enucleated fragments as it did in nucleated fragments is difficult to understand unless cytoplasmic synthesis or incorporation of isotopes utilizes the RNA originally formed in the nucleus. However, if synthesis of RNA independent of the nucleus occurs, the chloroplast, which is known to exhibit independent genetic properties, would be a likely site.

Also, a number of experiments show a long lag period before labeled RNA appears in the cytoplasm as contrasted to its appearance in the nucleus when cells are given a radioactive precursor of RNA (88, 90, 107). The study of incorporation by the hamster cells in culture indicated that the pool of RNA precursor could be effectively diluted. For example, addition of excess cytidine and uridine (50 times the molar concentration of that in the labeled medium), after removing cells from a medium with tritium-labeled cytidine, immediately stopped the increase in labeled RNA in the nucleus but not in the cytoplasm. Within 4 hr. essentially all of the labeled RNA of the nucleus had disappeared and an equivalent amount had appeared in the cytoplasm. In addition, the labeled nuclear RNA was more quickly dispersed into the cytoplasm when the nuclear membrane broke down at late prophase. However, the question of the sites of synthesis cannot be considered settled without much more data, perhaps with both heavy isotopes and radioactive isotopes as labels.

Division stages.—As already mentioned, DNA replication has not been observed during the division stages with the exception that in one species, *Tradescantia paludosa*, the replication extends into the leptotene stage of early meiotic prophase. However, it should be noted that during leptotene in this species the chromosomes are not highly condensed. DNA replication does not occur between the two meiotic divisions even in those species in which the nucleus reverts to a nearly typical interphase condition. As is well known, the chromosomes of many species remain condensed during this interkinetic stage and little if any RNA synthesis occurs.

In mitosis, RNA synthesis stops in mid-prophase and is not resumed until mid-telophase (90). In meiosis synthesis continues through most of the long prophase until the chromosomes become highly condensed in diakinesis (87, and unpublished). Protein synthesis continues through the division stages

in the cytoplasm of mammalian cells in culture (90), but in meiotic prophase of *Lilium* the uptake of isotope into proteins is very low compared to that which occurs in premeiotic interphase (88). On the other hand, many oöcytes make their greatest growth during meiotic prophase and these produce large quantities of RNA and protein. The chromosomes at such stages are very much expanded and frequently are not visible.

Measurements of oxygen consumption have shown a fall coincident with division stages in some types of cells. In the ciliate, *Tetrahymena*, measurements of oxygen uptake by cells induced to divide synchronously showed the drop (108). Erickson (22) noted a fall in the rate of oxygen consumption in whole lily anthers during the meiotic divisions and again at mitosis in the microspores. Stern & Kirk (75, 78), using anthers of *Trillium*, obtained similar results. Nasatir (60) later extended these findings to isolated microspores. Variations in oxygen uptake during the cleavage of frog eggs and eggs of various marine invertebrates have been reported but are so small that their significance has been questioned (5). Warburg (99) has reported certain changes in the oxidative systems of some tumors and has suggested a linkage with the rapid mitoses in such tissues.

One or more of three possible explanations can be offered for the various results: (*a*) the difference reflects an impairment in the cells' respiratory systems; (*b*) an energy reservoir is filled during interphase and used during mitosis; or (*c*) metabolism drops because less energy is required during mitosis.

A high rate of aerobic glycolysis associated with a lower rate of respiration has been reported for cancer cells (99). The suggestion has been made that an irreversible alteration in the oxidative system induced continued divisions and the resulting cancerous tissue. This idea has provoked much discussion and considerable work, with some contradictions and various explanations of the reported changes (5, 82). Any primary linkage between the type of respiration and the induction of cell division has not been demonstrated and the chance that one exists appears rather remote. The shift from respiration to glycolysis probably is characteristic only of certain tissues and in view of all of the known steps necessary for completion of the cell division cycle, such a change would seem insufficient to induce mitosis.

Swann's (82) experiments with sea urchin eggs showed that suppressing respiration up to a critical time delayed mitosis for a period equivalent to the interval of inhibition. After a critical stage (probably the beginning of prophase) mitosis proceeded in spite of the inhibition of respiration.

Bullough's (9) idea of an "antephase" build-up of an energy reservoir, which might appear supported by Swann's (82, 83) experiments, can as easily be explained by other means. Any progression of stages or synthesis of materials required for mitosis could be delayed by the inhibitors used. If the inhibition were reversible the delay would be proportional to the period of treatment. Studies by Gelfant (27, 28) on the mouse ear epidermis do not support the concept of an energy reservoir. The synthesis of the nucleic acids and various proteins of chromosomes must be accomplished prior to mitosis.

Likewise, evidence now indicates that the spindle proteins are formed before the actual division (88, 101). The cytoplasmic constituents also approximately double in amount during interphase, although the coupling of these processes with mitosis is perhaps less obligatory than the reproduction of the chromosomes.

Some cells may require less energy during mitosis, for there is no measure of the energy requirements for spindle organization and chromosome movement. The requirement could conceivably be smaller than the interphase growth requirements. There is no DNA synthesis during that period and RNA synthesis appears to stop. In rapidly dividing cells a large part of the protein is nuclear protein. If the synthesis of all of these components stops, even though the cytoplasmic protein synthesis continues, the energy requirements would be smaller unless compensated for by the needs of the division processes.

Stern (76, 77) has reviewed the evidence that sulfhydryl groups are particularly important in the division stages. He points out that the idea is an old one that arose from the observation of (*a*) a strong reaction of a number of proliferating tissues with nitroprusside, (*b*) the rise and fall in concentration of soluble thiols in relation to cleavage in sea urchin eggs, and (*c*) the inhibition of cells at the mitotic stages by thiol poisons and their reversal by cysteine, glutathione, and thioglycollate. Stern examined the soluble thiols in lily anthers during an 11-day interval surrounding mitosis in the microspores. He found a marked increase in total acid soluble —SH and —SS— up to division, and a fall afterward, most of which was glutathione, but no conversion of —SS— to —SH. He suggests a connection with the respiratory changes that likewise are associated with these division stages.

Physical and Chemical Agents Primarily Affecting the Interphase Stages

Temperature.—Variations in temperature within the physiological range of the organism produce changes in the rate of cell division. In *Lilium longiflorum* the first meiotic prophase requires about 7 days at 23°C. (93) and about 10 to 11 days at 20°C. (91). Some species have a rather narrow range of tolerance. In the root tip of *Vicia faba* changes in temperature in either direction from 20°C. were soon followed by a reduction in mitotic index. The number of cells in prophase, when taken as a percentage of the total, indicated that the delay was occurring just before the mitotic stages (7). In the ciliate, *Tetrahymena*, sublethal temperature shocks result in an accumulation of cells at predivision stages. Repetition of such shocks at appropriate times increases the synchrony of cell division until a large percentage of the population divide at nearly the same time (72, 108). Mammalian cells (strain HeLa) in culture are reported to become synchronized by lowering the temperature periodically to 4–6°C. (61). Presumably the delay is in interphase.

By using repetitive temperature and light cycles, the duration and intensity of which were matched to the synthetic capacity of the cells, James (43) induced division synchrony in *Amoeba proteus*, *Astasia longa* (62), and

Euglena gracilis. Similar types of treatments had been shown earlier to induce synchrony in cultures of *Chlorella* (57).

Radiation.—Variations in mitotic frequency are often associated with exposure to visible light, particularly in photosynthetic organisms. In ten species of green algae (Eugleninae) grown in a soil-water medium, mitosis was found to be restricted to a dark period following a day's exposure to daylight (50). Once initiated, the stimulus to mitosis was not reversed by light even though the cells had not reached prophase; therefore, the effect is on some interphase stage. Non-green species did not exhibit the response to visible light and one green species, *Euglena gracilis*, did not show light dependence when grown in a rich liquid medium.

Ultraviolet radiations delay mitosis in some cases, especially at wavelengths that are absorbed by nucleic acids. If the dose has not been too large the mitotic index falls slowly, with a gradual recovery (10). The interphase is the most sensitive stage, although cells at prophase are also affected.

Ionizing radiations affect various stages, but the most sensitive stage in many cells is preprophase (29). As prophase advances the cells become less sensitive, and after late prophase the division will occur following huge doses of x-rays (10). When relatively small doses are given there is a rapid drop in the mitotic index followed by an increase above normal as the cells stopped at late stages recover and reach division along with cells that were at earlier stages when irradiated. The processes affected by the radiation are not known. There is some indication that RNA metabolism may be involved since the nucleolus is by far the most sensitive structure. Microbeam irradiation with ultraviolet light for three minutes showed that mitosis was permanently stopped if the nucleolus was in the field of exposure. Irradiating other parts of the nucleus to the same extent produced delay but did not usually prevent cell division. After middle prophase the cell becomes less sensitive and finally at late prophase is insensitive to this amount of radiation applied to the nucleolus (26). Since the nucleolus is a very active site involved in RNA metabolism and the synthesis stops about the time the sensitivity drops, RNA metabolism is perhaps a good choice for a sensitive process. However, Gaulden (25) has shown that the mitotic delay produced by low doses of x-rays is reversed by hypertonic salt solution. Since the visible change involved is the degree of condensation of the chromosomes, the basic processes affected remain obscure.

Numerous studies have indicated that DNA synthesis is quite sensitive to radiation. Many of these studies have measured effects on masses of cells or whole tissues. In such cases the suppression of mitosis at any stage would result in a drop in DNA synthesis as the cells failed to proceed to the stage where replication begins. The replication process actually is not especially sensitive to radiation damage, for studies on the incorporation of labeled thymidine into DNA show that synthesis continues after relatively large doses of ionizing radiation. Recently some evidence has been obtained that radiation may actually increase the rate of synthesis in actively dividing tissues such as the root meristem (18). This is perhaps not unreasonable since

Kornberg and his collaborators have found that slightly damaged DNA is a better primer for the enzymatic synthesis of DNA in vitro than native DNA (46).

Chemical agents.—Maleuric acid (74b) has been shown to produce mitotic abnormalities and depress the mitotic index in both plant and animal cells. Studies with tritium-thymidine to measure progress of cells through the cycle indicated that the interruption occurs both in interphase and at division stages.

The antibiotic, actidione, produces a delay in the division of root cells (95). Treatment for 15 min. in 2.52×10^{-5} M solution produced a 5-hr. delay. The mitotic index was rapidly reduced to zero, but after a delay the division rate was restored when the drug was removed. The most sensitive stage appears to be preprophase or some stage just prior to that. After prophase has started, cells will go on through division in the presence of sublethal doses.

Brachet & Ledoux (6) showed that immersion of cleaving eggs at the morula stage in a solution of ribonuclease stops all mitoses in the external layers. Cells already in division at the beginning of treatment showed various abnormalities, but most of the cells were blocked in interphase. Treatment of ascites tumor cells, fibroblasts in culture, and several other types of cells produced a marked reduction in mitotic index (12, 49). Chèvremont and co-workers (13) have also shown that deoxyribonuclease inhibits mitosis and has some peculiar effects in which there is an accumulation of DNA in the mitochondria. McLaren, Jensen & Jacobson (55) have also shown that barley root cells rapidly absorbed ribonuclease, lysozyme, and human hemoglobin when grown in solutions of the proteins.

Halogenated pyrimidines have been used extensively in recent experiments on the inhibition of tumor growth. Karnofsky & Basch (44) applied some of these substances to the sand dollar embryo. Embryos are stopped by 5-fluorodeoxyuridine in early blastula. An excess of thymidine or bromodeoxyuridine protects the embryo, but those treated with bromodeoxyuridine later die. These compounds and other analogs of purines and pyrimidines affect the synthesis of DNA or RNA, or both types of nucleic acid, and therefore would be expected to eventually block mitosis through an effect on the interphase stages. Djordjevic & Szybalski (21) reported that human cells (HeLa) in culture grow and divide indefinitely when bromodeoxyuridine is added to the medium at a concentration of 3×10^{-5} M. Bromodeoxyuridine can replace up to 45 per cent of the thymidine in the DNA when given at higher concentrations. Cells also grow for limited periods in iododeoxyuridine, but prolonged growth results in loss of viability. Cheong, Rich & Eidinoff (11) found that another type of mammalian cell would grow for a time in a medium containing bromodeoxyuridine from 5 to 100 μM. On the average, at 75 μM the cells underwent one division and stopped. They also prepared tritium-labeled bromodeoxyuridine which labeled nuclei sufficiently for autoradiography.

Nutritional and hormonal factors.—Wilson and collaborators (103) have

studied the mitotic activity in excised pea roots. When maintained in balanced salt solution, mitosis drops to 10 to 15 per cent of that of controls and remains at this level for about 48 hr. A variety of carbon sources, including sucrose, glucose, fructose, dihydroacetone, and glyceraldehyde, restore part or all of the activity for periods of several hours to nearly two days. The effect can be reversed by some respiratory inhibitors.

Das, Patau & Skoog (16) have shown that mitosis and cell division can be induced in excised tobacco pith by the use of kinetin and indoleacetic acid (IAA). In a study of DNA synthesis at the cellular level by cytophotometry, Patau *et al.* (64) showed that DNA doubling was induced first, followed later by mitosis. In control cultures little or no DNA synthesis occurred. The two substances were effective alone, but most stimulation occurred when both were used together. Later, Das *et al.* (17) used thymidine-H^3 to detect the synthesis of DNA in the cultures. In control cultures incorporation occurred in about 20 per cent of the nuclei during the first two days, but few if any were noted thereafter. After two days IAA induced DNA synthesis, but kinetin did not. Continued cell reproduction was maintained only in the presence of both.

In a study of the effects of IAA and kinetin on recovery from the mitotic inhibition produced in irradiated cells of root meristems, Guttman & Brown (31) reported recovery with IAA if the cells were irradiated at or just prior to prophase but no recovery with kinetin. Kinetin and IAA together produced complete recovery under the conditions used by Guttman & Brown regardless of the time in the cell cycle at which irradiation occurred. When the two substances were used together the frequency of chromosomal abberations was also reduced. IAA had no effect when used alone, but kinetin stimulated rejoining of breaks for cells irradiated when close to mitosis.

By the use of radiation and chemical agents Haber & Luipold (32) obtained evidence for different mechanisms initiating cell division and cell expansion. In germinating lettuce seeds cell division and cell expansion usually coincide in time. Gamma-irradiation plus kinetin treatment at 26°C. delayed germination, but recovery and germination occurred several days before cell division began. Growth at 10°C. also produced a similar effect on the beginning of the two processes. At 26°C. in a solution of manitol, cell division preceded germination by many days, while at 30°C. only a small percentage of seeds germinated, but considerably more of the seedlings had mitoses. They concluded that kinetin stimulated cell expansion but not cell division in lettuce seed.

Harding, Donn & Srinivasan (33) have used tritium-labeled thymidine to visualize the wave of premitotic events that radiate out from the point of injury in the lens epithelium of the rabbit. The stimulus for mitosis and the events leading up to it move out from the site of the wound so that an arc or circular group of cells are replicating DNA simultaneously. Indications for a more specific hormonal control of mitosis was obtained by Stich (81). The effects of sera and homogenates on the mitotic rate in regenerating liver and

homologous and heterologous tissues in baby rats were studied to gain information on organ-specificity of the substances. Sera from adult rats inhibited the onset of mitosis and synthesis of DNA, whereas sera taken from rats 24 to 92 hr. after partial hepatectomy, from rats bearing a Walker carcinoma, or from rats with induced hepatomas, stimulated mitosis in regenerating liver. Homogenates of fully grown rat liver decreased mitotic rate, and homogenates of hepatoma and carcinoma increased mitotic rate in regenerating liver. The experiments on young rats suggested that organ-specific inhibitors of mitosis may take part in regulating the mitotic rate.

Agents Affecting the Division Stages

No new chemical agents primarily affecting the division stages seem to have been reported. However, Mazia and collaborators (52, 53) have used mercaptoethanol for some revealing studies on mitotic inhibition. According to their account the cells of marine eggs can be blocked in metaphase by this sulfhydryl compound. The reaction is reversed when the eggs are removed and division proceeds. If eggs of the sand dollar are blocked for an appropriate time at the first division, a tetrapolar spindle is formed after removal. The next division is abortive since only monopolar spindles form. Chromosome counts show that each of the four cells after division by the tetrapolar spindle have the full complement of chromosomes but measurements of DNA in the four cells indicate that no increase occurred during the delay. If these observations and measurements are all correct, chromosomes can divide in these cells without replication of DNA. Mazia's interpretation is that the mitotic centers divide in the presence of the mercaptoethanol and get out of phase with the chromosomes, but as a result of the monopolar abortive division that follows, the two get back in phase.

The Spindle and Associated Structures

Improvements in equipment and new biological materials have resulted in advances in the birefringence studies of the spindle in living dividing cells. Bajer (3) has worked out a technique for studying mitosis in endosperm cells in extremely flattened preparations. Not only has he produced elegant cinemicrographic pictures of the process, but excellent pictures with polarized light give a clearer picture of the crystallization patterns in spindle formation and function. Inoue's (40, 41) studies indicate that both kinetochores (centromeres) and centrioles are centers for crystallization. These centers are active in all directions but the activity of the kinetochores is ordinarily polarized. The spindle micelles crystallize into filaments from either pole or between two poles and form astral rays and spindle fibers. The aligned micelles are suspended in a pool of randomly oriented particles which are more hydrated. The oriented micelles and the unoriented material are in equilibrium. He believes the oriented micelles to be crosslinked to some degree, but the amount of crosslinking is a sensitive function of pH and ionic strength. The various mitotic movements of chromosomes are explicable in terms of

changes in the delicately balanced equilibria which also involves shifts in water balance. Fixation and isolation would readily shift either the orientation equilibrium or crosslinking or both.

Fixed cells of plant root tips with excellent preservation of membranes and various cytoplasmic structures do not show any fibers in the spindle (65, 102). Tubular structures in association with the spindle are part of the endoplasmic reticulum. Their function perhaps is connected with the reformation of the nuclear membrane rather than the movement of chromosomes.

Sato (71) recently published electron micrographs showing bundles of fibers attached at the centromere of chromosomes and oriented along the axis of the spindle in microsporocytes of *Lilium*. The material was fixed in a mixture of one part 0.1 *M* cadmium chloride and one part absolute alcohol at pH 4.4. It is not clear whether these fibers represent an enhanced aggregation of the oriented micelles that Inoue's results indicate to be present, or a better representation of the actual structure of the spindle. However, one would prefer a fixative that would preserve other parts of the cell in better condition along with the spindle.

Cell and Organ Culture

Although animal cells in culture have proven excellent objects for studies of cell division, plant cells in tissue culture are less suitable for various reasons. Their tendency to clump and the rigid cell wall are handicaps. The failure of such cells to adhere to glass surfaces reduces the conditions under which they can be handled and observed. The most interesting developments in this field are the demonstrations that single somatic cells can give rise to whole plants. Muir, Hildebrandt & Riker (58) grew tissues on a shaker in liquid medium. With friable types of tissue cultures of marigold, *Nicotiana tabacum*, and grape they obtained single cells and clumps of cells. Cells, isolated and placed on filter paper that was applied to the freshly cut surface of an established piece of tissue, divided and developed in a few instances into sizable clumps of cells. Braun (8) used their technique to isolate clones from single cells of tobacco crown gall tissue that had been freed of bacteria and grown for several years in culture. The crown gall was the type that produces very abnormal buds and leaves in culture. Braun grafted the abnormal organs formed from these cells onto healthy tobacco plants. After one or two transfers he obtained rather normal branches with flowers, and from these he obtained seeds. These seeds produced normal *Nicotiana tabacum* plants like the ones from which the original cultures were established. He interpreted the change as the loss of a self-duplicating cytoplasmic particle that had maintained the tumor character. Because of the rapid divisions induced in the bud meristems (much faster than the cells ever divide in culture) Braun concluded that the nuclear and cell division proceeded faster than the rate of reproduction of the particle, and that therefore cells eventually divided without distributing the particle to one of the daughter cells.

Stewart *et al.* (79, 80) also obtained isolated cells in liquid cultures from

carrots. He observed these divide and form clumps of cells. Whole normal carrot plants were eventually produced from similar clumps.

These experiments indicate that plant cells capable of division usually retain the genetic capacity to undergo differentiation and regenerate the whole plant. Braun interprets his experiments to mean that the crown gall cells have acquired some self-reproducing particle, presumably in the cytoplasm, that causes the change to gall formation. Because of the rapid divisions induced in grafts, some cells of the population lose the particle because its reproduction does not keep pace with mitosis. This, of course, is the most likely explanation, but one must keep in mind that cells can become infected with viruses by means of grafts. Also, perhaps unknown viral-like agents reside in the cytoplasm or become integrated into the nuclear genetic material as has been described for bacteria [Jacob, Schaeffer & Wollman (42); Lederberg (48)]. Although such a system is unknown in higher forms, indications of a comparable relationship have been reported in mammalian cells by Vogt & Dulbecco (98). In such a situation cells might either lose or gain the ability for differentiation at either the nuclear or cytoplasmic level of organization.

Studies of meiosis using cell cultures have been only moderately successful. Since cells can not yet be brought through the long prophase of the first meiotic division, isolated cells have little value beyond facilitating studies of the mitotic spindle and features of the late stages of division. The next best technique is to culture the excised anthers under conditions in which meiosis will be completed in a regular fashion. So far the limitations in this work have not allowed regular survival of meiotic cells in anthers excised earlier than late leptotene. Even when anthers are removed while they have cells at this stage, the microsporocytes often abort or various anomalies appear when the anthers seem to survive. Such cultures are of some use, but since the greater part of the synthesis of materials for development of microsporocytes occurs before leptotene, many interesting physiological studies are excluded. A few papers have appeared indicating improvement in growth with the addition of the growth substances, IAA and kinetin, to basal tissue culture media (96). In addition, nucleic acids were reported to enhance the survival of anthers in culture (97). The general applicability of any of these substances is very dubious, however. Lily anthers with sporocytes at late leptotene advance far enough so that the sporocytes often divide on basal media. The addition of the substances mentioned above, combined with various nucleic acid derivatives and amino acids, produced little effect (47). Metabolism in the sporocytes appears to be very slow in prophase (88) and perhaps the greatest advantage comes from maintaining the growth and differentiation of the tapetal cells. However, the whole problem in this type of organ culture is to maintain differentiation rather than to induce cells to divide, as is the goal in most tissue culture. The maintenance of this differentiation has proven more difficult than keeping proliferating tissues dividing.

Comments and Conclusions

An attempt to write a review of mitosis and meiosis, subdivided on the basis of the events in the cell division cycle, points out the paucity of studies that are primarily concerned with this aspect of physiology. At times the subdivisions seem arbitrary and sometimes they are impossible to make. However, the fact that it has become possible to arrange data in relation to the cell cycle indicates a trend in modern cell physiology. Of course, some features of cellular metabolism may have little relation to the division cycle, but just as studies at the cellular level have revealed features of cellular metabolism not heretofore realized, so should studies of metabolism in relation to the whole cycle add considerably to our understanding.

The development of techniques for measurements at the cellular level and the increasing use of methods for synchronizing cells in division make the collection of data on this basis feasible. The use of radioisotopes and autoradiography at cellular and subcellular levels adds an important tool for kinetic studies of cell division and growth. One may safely predict that by the time another review is written, a better arrangement in terms of the cell division cycle will be possible. Some of our concepts will no doubt have to be modified or changed drastically to accommodate the new data, for this point of view is relatively new in physiology and the available data are frequently insufficient for generalizations. It is this reviewer's hope that the trend will continue and that physiologists, particularly those who utilize plant cells, will direct their efforts more along these lines.

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INDOLE AUXINS[1,2]

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Introduction

Various aspects of auxin physiology have been reviewed in this series (1, 14, 70, 74, 81, 165, 189) and elsewhere (114, 119, 133, 142, 180, 191), and the recommended nomenclature for auxins (208) has now come into general use. The aim of this review is to emphasize the physiological importance of indole auxins as indicated (*a*) by their occurrence in the organs of normal and abnormal plants, (*b*) by the auxin precursor—free auxin—bound auxin status of indoles in tissues, and (*c*) by their participation in the biochemistry of the plant. In the discussion of these topics, which incorporate the biogenesis, metabolism, and utilisation of indole auxins, an attempt will be made to establish the existence of a regulatory system that accounts for the balance of indole auxins observed in plants.

Although many plant physiologists are turning to attractive fields of study involving gibberellins and kinetins, and interactions of these with indole auxins, such interactions will not be discussed here.

Auxins in Normal Plants

On the basis of chromatographic evidence, IAA was reported in more than twenty plant species (14). Later studies have revealed IAA in onion leaves (39), mustard leaves (31), mistletoe berries (82), pea and gorse nodules (146), crown-gall of tomato (41, 52), orchid pollen (135), wheat root (116), lentil root (150), pear and maple buds (199), lettuce seedlings (68), and tomato plants (220). It is desirable that these chromatographic identifications should be consolidated by isolations of IAA. The extraction and characterisation of IAA for the first time from a vegetative plant, namely, cabbage [Post (156)], and the earlier isolation of IAA from maize kernels [Haagen-Smit *et al.* (83)], represent the only proofs of its accepted widespread occurrence.

Whilst there are also reports that other acidic indole auxins such as β-(indole-3)propionic acid (61, 121, 131), γ-(indole-3)butyric acid (23), IPyA (23, 192, 193), indole-3-glyoxylic acid (61, 131), and indole-3-glycollic acid (61, 131) can be extracted from plants, further work and discussion lead to the conclusion that it is improbable that free IPyA and indole-3-glycollic acid have been obtained from plants (18, 80, 97, 130, 176, 190). For example,

[1] The survey of literature pertaining to this review was concluded in July 1960.

[2] The following abbreviations will be used: IAA (3 indoleacetic acid); IAAld (indole-3-acetaldehyde); IAN (indole-3-acetonitrile); IPyA (indole-3-pyruvic acid).

chromatography in ammoniacal solvents was employed to demonstrate IPyA in maize endosperm [Stowe & Thimann (192, 193)]; complete breakdown of synthetic IPyA during chromatography, however, was observed in such solvents and Bentley *et al.* (18) therefore concluded that IPyA in maize cannot be demonstrated by this method. Although the metabolism of tryptophan in watermelon slices was considered to yield IPyA which did not undergo complete breakdown when chromatographed in ammoniacal solvents [Dannenburg & Liverman (49)], Kaper & Veldstra (97) considered that such identification was not convincing. The spot on the chromatogram taken as IPyA (49) corresponded in R_F and colour reaction (Ehrlich) to a spot that, on further chromatographic and spectroscopic examination, did not represent IPyA (97). It seems that separation and recognition of the ketonic and enolic forms of IPyA on chromatograms [Schwarz & Bitancourt (177)] and chromatography in other solvent systems (108) may provide a new approach to these problems.

Among the neutral indole auxins, IAN is recognised as especially important. It was first isolated from cabbage [Jones *et al.* (87, 95)] and chromatographic evidence has subsequently indicated its occurrence in numerous plants (14, 218). The release of IAN from a water-soluble, ether-insoluble precursor obtained from cabbage (25, 91) has been confirmed (156).

Although IAAld has not been isolated from plants, a substance with properties similar to the synthetic compound has been detected in maize [Yamaki & Nakamura (226)] and in pea, but not cabbage [Larsen & Aasheim (115)]. There appears to be no reason for thinking that these specimens of IAAld and the new indole-3-pyruvaldehyde isolated from cabbage [Post (156)] are the same substance. However, the increased auxin activity obtained when IAAld is oxidised by Larsen's soil technique (115) is possibly not specific for IAAld, and indole-3-pyruvaldehyde may react similarly.

The isolation of the highly active auxin ethyl indole-3-acetate from immature maize seed was reported [Redemann *et al.* (166)], but since an extraction process employing ethanol was used, it was suggested [Henbest *et al.* (87)] that the ester obtained was an artifact formed from the IAA known to be present (83). When the extraction was carried out using ether, no ethyl indole-3-acetate was obtained [Fukui *et al.* (67)], but extraction with ethyl acetate gave a product interpreted as ethyl indole-3-acetate [Hinsvark *et al.* (89)]. The chromatographic identification of ethyl indole-3-acetate extracted with ether from apple endosperm [Teubner (201)] has also been questioned [Luckwill & Powell (125)]. However, the neutral auxin extracted by water from peach buds not only had a suitable R_F and suitable chromogenic reactions, and high activity in the pea and oat section tests, but was also converted to IAA by alkaline hydrolysis and destroyed by acid hydrolysis. It was hence regarded as ethyl indole-3-acetate by Blommaert (24). Likewise, the neutral auxin extracted from mistletoe berries by hexane [Guern (82)] had properties similar to esters of indole acids (59, 82); the occurrence of ethyl indole-3-acetate has also been indicated in other plants (14).

Apart from indole auxins a number of indole auxin precursors have also been extracted from plants. Thus, tryptophan occurs in potato (197), lupin (140), strawberry (138), barley (154), Brussels sprouts (107), tomato (220), and in other plants where its level appears adequate for IAA production (191). Tryptamine has been isolated from *Acacia* (221), and has been detected in tomato (219, 220) and maize (86). The indole auxin precursor ascorbigen (or a bound form of ascorbic acid) has been extracted from various *Brassicae* (71, 107, 111, 127, 144, 158, 159, 172, 182, 209), *Citrus* (7) and members of the family Papilionaceae (10).

Several of the indole compounds that occur in plants are inactive or possess only slight auxin activity. They are regarded as intermediates in auxin biogenesis or as products of auxin detoxification and degradation. These have relatively simple structures like indole from orange blossom (194); skatole from various *Brassicae* (121) and *Tachigalia myrmecophila* (4); indole-3-aldehyde from *Brassicae* (50, 96) and pea (36); indole-3-carboxylic acid from tomato (41, 52), pea (36), and various *Brassicae* (36, 61, 96, 120, 131, 158); malonyltryptophan from tomato, pea, and oat (72); 5-hydroxytryptamine from cotton fruit and skunk cabbage (34), tomato (220), and cowhage (28); and 5-hydroxyindole-3-acetic acid from tomato (220). Other indole compounds such as gramine, abrine, hypaphorine, and bufotenine (191), and the 30 more complex indole alkaloids extracted from various plants (196), also help to indicate the ubiquitous nature and the scope of the metabolic processes that function with indole derivatives.

Auxins in Abnormal Plants

Many parasitic microorganisms cause plants to exhibit such symptoms as tissue proliferation and cell elongation. The suggestion that excessive levels of IAA occur in these abnormal plants (29, 81) has been confirmed in some instances by chromatographic evidence (41, 102, 148). In the present discussion of indole auxins and auxin precursors found in abnormal plants, the term "abnormal" is also applied generally to plants which have been treated, or given environmental conditions, different from those of control plants.

The idea that IAA causes the distortion of root hairs of leguminous plants after infection by nodule organisms (*Rhizobium* spp.) [Brian (29)] has been supported by the finding that IAA is the dominant auxin in pea root nodules, and that levels of extractable auxin are much lower in root tissues than in nodules [Pate (146)]. Formation of tumors initiated by the fungus *Synchytrium endobioticum* in potatoes resulted in the accumulation of indole auxins in the peripheral fast growing parts, and the tumor tissue contained more auxin than normal tissue [Reingard & Pashkar (168)], although with tumor-prone varieties the epidermal tissue also showed greater ability to destroy IAA (145).

The higher auxin content reported in extracts from tumor tissue initiated by the crown-gall organism *Agrobacterium tumefaciens* (88, 105, 118) was

further elucidated by chromatographic evidence (chromogenic reactions and bio-assay) that IAA was detectable in crown-gall tissue of tomato stem but not in normal tomato stem [Clarke *et al.* (41, 52)]. Furthermore, there is evidence that in sunflower crowngall the extra IAA needed to transform incipient tumor cells to fully altered tumor cells may not be bacterial in origin but due to the action of an inhibitor of the IAA-oxidase [Lipetz (122)].

Auxin production by many parasitic fungi either in culture or in the host has been reviewed [Gruen (81)]. More recently, chromatogram-bioassay has provided evidence that the extracts from leaves and stems of *Verticillium*-infected tomato plants contained approximately twice as much activity in the IAA zone as healthy plants [Pegg & Selman (148)]. It was suggested that the major part of the *Verticillium* syndrome, including petiolar epinasty, tylosis, pith hyperplasia, and the formation of adventitious roots, results from this.

Similarly, in *Peronospora*-infected plants (*Capsella* spp.) it was established by chromatography that increased levels of IAA and IAN were present. These were taken as the cause for the hypertrophy and curvatures seen—as well as for the changeover from normal prosenchymatic bast cell to thin wall parenchymatic cell production [Kiermayer (102)].

Chromatographic study of extracts from virus-infected and healthy Burgundy vines showed a marked difference in the pattern of chromogenic spots (indoles) but levels of organic acids, amino acids and sugars were not changed [Bruckbauer & Pioth (33)]. Similarly, bioassay of chromatograms showed that leaves on tobacco plants infected with mosaic or *X* virus contained less IAA than leaves on healthy plants [Beauchamp (11)]. Diminished concentrations of auxin in nearly all parts of the plant in comparison with healthy plants of the same age were also reported [Pavillard (147)]. This effect was also noted in tomato and potato plants infected with the potato *X* virus where the deficiency in auxin was likewise associated with stunting.

Some reports indicate that the auxin content of plants is affected by the quality and duration of the light they receive. Evidence has been put forward for a simple relationship, such as the lower amount of IAA in Biloxi soybean plants receiving 18-hour photoperiods than in plants receiving 8-hour photoperiods, although such a difference was not found with Lincoln soybean [Vlitos & Meudt (214)]. Likewise, the IAA content of oat seedlings increased with red or far-red light treatment (22). A more complex relationship, however, emerges from the comprehensive studies using pea (149) and *Sinapis alba* (31, 32). Thus, the amount of endogenous auxin found in pea seedlings grown in the dark, or in red light, was found to change with age. The plants in red light had less auxin than those in the dark in the first few days of growth, about the same amount after 8 to 11 days, and distinctly more after 21 days. On some chromatograms the auxin activity not only corresponded to the IAA position but was also Ehrlich positive [Philips *et al.* (149)]. From the way that the IAA content (determined both as free and bound IAA) varies with the photoperiod in *Sinapis alba* grown under phyto-

tron conditions, it was concluded that not merely the metabolism of one substance, a flowering hormone, but the entire metabolism is affected by the photoperiod [Bronchart (32)].

Non-vernalized lettuce seedlings were found, by a chromatographic study, to have twice the level of free IAA as vernalized seedlings [Fukui *et al.* (68)].

Apart from IAA, the level of auxin precursors also altered with environmental conditions. Thus, the level of ascorbigen and IAN was higher in *Brassica oleracea* seedlings grown in the light than in plants grown in the dark, but the latter contained higher levels of tryptophan [Kutáček *et al.* (107)]. This relationship changed very little over several days, and in view of the biosynthesis of ascorbigen from tryptophan [Kutáček *et al.* (108)], it would be of interest to know whether the balance of these important precursors in other plants is changed with the lighting conditions.

Decreased levels of auxin may also have a genetic basis. Thus, greater amounts of diffusible auxin were obtained from diploid red clover apices than from autotetraploid apices (48) whilst albino tobacco plants had a diminished auxin content relative to normal plants (147). Tobacco plants exhibiting the physiological disease known as "frenching" gave one quarter the auxin (with same R_F as IAA) as normal plants, and it is suggested that the symptoms result from auxin deficiency [Kefford (100)].

An indirect link between indole auxins and the nutrients available to the plant was given by the observation that the tryptophan content of barley proteins was decreased by 45 per cent as a result of a reduced N supply, whilst a somewhat less severe drop arose from P or K deficiency [Pleshkov (154)].

Increased levels of extractable auxin have been obtained after plants or plant tissues have been chemically treated. For example, in *Trifolium ochroleucum* (152) and pea seedlings (149) sprayed with gibberellic acid, oat coleoptiles treated with α-(4-chlorophenoxy)-isobutyric acid (66), and wheat roots grown in nutrient solution in which the pH was raised from 5 to 7.5 for an hour (170) the evidence indicated an increased auxin content which could have been IAA. Similarly, the application of a chlorohydrocarbon formulation to break the dormant period in potatoes caused profound changes, including an increased indole-auxin content and a decrease in the level of inhibitors (198, 210, 211). The increase in IAA content correlated with the level of tryptophan in the tubers [Szalai (197)].

Chemical treatment with maleic hydrazide caused a decrease in IAA content of onion (207) but it has little effect, except at very high concentrations, on IAA levels in many growing plants (8). Similarly, the suggestion that aryloxyacid herbicides operate by disturbing the level of endogenous auxin has not been supported by experimental evidence [Audus (8)].

Unidentified Indole Auxins

Facts about unidentified auxins occurring in plants have continued to accumulate and some of these substances may be regarded as indole com-

pounds. They are included in Table I for the following reasons: They either give chromogenic reactions with reagents usually reactive with indole compounds—these include the Ehrlich (94), Salkowski (171), Gordon & Weber (77) and nitric-nitrous acid (59) reagents—or they are compounds which, on examination by chromatography and bioassay, exhibit characteristic physiological properties. Thus, accelerator α [Bennet-Clark & Kefford (12, 99)]; Pinus 1 auxin [Fransson (64)]; auxin-*a* from wheat root [Lexander (116)]; and an auxin from lentil root [Pilet (150)]; all promote cell elongation in roots as well as in shoots and hence in this respect behave like α-(indole-3) isobutyric acid (57, 84, 139).

The possibility that accelerator α, which has been extracted from several plant species (12, 98, 99), might be Kögl's auxin-*a* (12) has not been supported, and since the eluted accelerator, hydrolysed by 7 *N* sodium hydroxide, does not yield IAA it is not likely to be indoleacetylaspartic acid (13). The inhibition of cress root growth by IPyA (18) also indicates that accelerator α may not be IPyA as postulated (192). Housley & Bentley (91) consider that this accelerator may be an artifact formed during the preparation of plant extracts for chromatography.

The *Aegopodium*-rhizome auxin (Table I) initially regarded as probably IAN (12) failed to yield any IAA by alkaline hydroysis (13) and in this and other respects it appeared similar to the neutral auxin reported in tobacco (216, 217) and the auxin-*Z* from algae (15). The neutral auxin, X_2, from maize, was also considered to be different from ethyl indole-3-acetate IAN, or IAAld (193).

In the continuous culture of excised rye roots it was found that tryptophan was not effective if added aseptically to the medium after sterilisation, but it was effective if present during the autoclaving process. The results were not explicable in terms of IAA or IAN formation [Roberts & Street (169)]. This effect was also observed with cultures of carrot phloem explants grown on various media and it was suggested that a substance from heat-treated tryptophan stimulates cell proliferation [Shantz & Steward (183)].

In general, the chromogenic reactions (e.g., Ehrlich, Salkowski, etc.) carried out with the unknown auxin still on the chromatogram are not so sensitive as the oat, wheat, and pea bioassays with the segmented chromatogram, and possible sources of error in the interpretation of the results also demand extra caution (27). Some indole compounds give a negative or only a slight chromogenic reaction (91), and certain phenols and polyphenols can interfere with the assessment of IAA by the Salkowski test (153). Also, a number of naturally occurring substances, not indole compounds, are reported to give similar chromogenic reactions on chromatograms. For example, certain sugars (26, 180), phenols (180, 188, 194), and leucoanthocyanins (137) can react like indole compounds but sometimes the colours are transient (41, 52), whereas most indole compounds give fairly stable dyestuffs on the paper (59, 94, 180, 181, 193). However, the conclusion (based on results with Ehrlich's reagent) that indole compounds are present

TABLE I

UNIDENTIFIED INDOLE AUXINS DETECTED BY PAPER CHROMATOGRAPHY

Name	Source	Solvent system*	R_F	Bio-assay	Chromogenic reaction†	Literature
X_3	Maize seeds	*i*P/A/W 10 1 1	0.83	Oat coleoptile extension Pea stem curvature	S = transient blue-green	(193)
X_2	Maize seeds	*i*P/A/W 10 1 1	0.52	Oat coleoptile extension Pea stem curvature (inactive)	S = brown-red	(193)
X_1	Maize seeds	*i*P/A/W 10 1 1	0.12	Oat coleoptile extension Pea stem curvature	S = crimson. IPyA suggested	(193)
—	Maize seeds	*i*P/W 4 1	0.20–0.40	Oat coleoptile extension	S = red in alk. solvents S = purple in other solvents E = purple	(53)
e	Maize seeds	H/W (Satd.)	0.30–0.45	Oat coleoptile extension	E = blue violet	(86)
f	Maize seeds	H/W (Satd.)	0.00–0.25	Oat coleoptile extension	E = positive. Tryptamine suggested	(86)
g	Maize seeds	H/W (Satd.)	0.80–1.00	Oat coleoptile extension	E = positive	(86)
a	Maize seeds	*i*P/A/W 50 7 3	0.00–0.10	Oat coleoptile extension	E = positive	(86)
—	Maize, immature seed	—		Oat coleoptile extension	IAA-arabinoside suggested	(189)
—	Lettuce, germinating seeds	*i*P/A/W 10 1 1	0.30	Oat coleoptile extension	S = weakly positive E = weakly positive	(155)
—	Lettuce, germinating seeds	*i*P/A/W 10 1 1	0.40–0.60	Oat coleoptile extension	S = weakly positive E = weakly positive	(155)
—	White mustard leaves	*i*P/A/W 80 5 15	0.73 0.43 0.28	Oat curvature	Indole compounds suggested	(31)
E	Kale	*i*P/A/W 80 5 15	0.64	—	N = red. Indole-3-glycollic acid suggested	(61)

* The solvents employed were: W = Water, A = 0.880 Ammonium hydroxide, *i*P = *iso*propyl alcohol, H = *n*-Hexane, B = *n*-Butanol, E = Ethanol.
† The reagents employed were: E = Ehrlich, S = Salkowski, G = Gordon & Weber, N = Nitrous/Nitric acid.

TABLE I *Continued*

Name	Source	Solvent system*	R_F	Bio-assay	Chromogenic reaction†	Literature
B	Kale	*i*P/A/W 80 5 15	0.45	—	N = transient red	(61)
—	Savoy stem	*i*P/W/A 80 15 5	0.77	Oat coleoptile Pasten test	IAAld suggested	(120)
—	Potato peelings	B/A/W	0.40–0.42	Oat coleoptile extension	γ-(indole-3) butyric acid suggested	(23, 24)
—	Potato + Rindite	*i*P/A/W 10 1 1	0.24	Oat coleoptile extension	IPyA suggested	(210)
—	Potato + IAA	W/E 3 7	0.63	Oat coleoptile extension	S = crimson-rose E = rose	(145)
—	Potato	B/A/W	0.05–0.15	Oat coleoptile extension	IPyA suggested	(23)
X	Barley leaves	*i*P/A/W 10 1 1	0.87	Pea root extension	S = positive G = brown	(179)
2	Oat coleoptile tips	*i*P/W/A 85 15 5	0.25	Oat curvature Oat coleoptile extension	Addition compd. of IAA suggested	(162)
Ribes$_2$ *Auxin*	*Ribes nigrum* berries	B/A/W 25 2 25	0.35–0.45	Wheat coleoptile extension and coleus petiole abscission	G = red after 4 hr.	(224)
Ribes$_3$ *Auxin*	*Ribes nigrum* berries	B/A/W 25 2 25	0.88	Wheat coleoptile extension and coleus petiole abscission	E = grey. IAN suggested	(224)
—	*Ribes grossularia* berries	B/A/W 25 2 25	0.40–0.45	Wheat coleoptile extension	G = pink	(224)
Ribes A	*Ribes nigrum* seeds	*i*P/A/W 80 15 5	0.10–0.20	Wheat coleoptile extension	E = blue-violet	(103)
Ribes C	*Ribes nigrum* seeds	*i*P/A/W 80 15 5	0.75–0.85	Wheat coleoptile extension	E = red-violet	(103)
Ribes D	*Ribes nigrum* seeds	*i*P/A/W 80 15 5	0.85–0.95	Wheat coleoptile extension	E = violet Ethyl indole-3-acetate suggested	(103)

TABLE I *Continued*

Name	Source	Solvent system*	R_F	Bio-assay	Chromogenic reaction†	Literature
—	Calimyrna fig fruit	*i*P/W/A 10 1 1	0.20–0.32	Coleus petiole abscission	S = faint pink	(47)
Malus 1	Fruit and seeds	B/A/W 25 2 25	0.05–0.10	Wheat coleoptile extension	E = purple. Accelerator *α* suggested	(124)
Malus 3	Leaves and seed	B/A/W 25 2 25	0.75–0.95	Wheat coleoptile extension	E = pale purple	(124)
N_2	Mistletoe berries	*i*P/A/W 10 1 1	0.85	Oat coleoptile extension	E = blue/blue-grey N = yellow-orange	(82)
"IAN"	Ground elder rhizome	*i*P/W 10 1	*ca.* 0.90	Oat coleoptile extension	N = blue green	(12)
—	Ground elder rhizome	*i*P/W 10 1	*ca.* 0.80	Oat coleoptile extension	Not hydrolysed by alkali like IAN	(13)
A_1	Pea nodule	*i*P/A/W 80 5 15	0.75–0.85	Oat coleoptile extension	—	(146)
—	Tobacco leaves and buds	*i*P/A/W 10 1 1	0.98	Split pea curvature Tomato epinasty	E = purple	(216)
		W	0.67	Oat curvature Oat coleoptile extension	S = red brown	(217)
Z	Algae	*i*P/A/W 10 1 1	0.8–1.0	Oat coleoptile extension	E = red/purple S = pink N = pink	(15)
X	Algae	*i*P/A/W 10 1 1	0.3–0.5	Oat coleoptile extension	Convertible to *Z*	(15)
Accelerator *α*	Several	*i*P/W 10 1	0.0–0.3	Oat coleoptile extension Pea root extension	—	(98) (99)
—	Lentil root	*i*P/W/A 80 10 10	0.05–0.15	Lentil root extension Lentil shoot extension	—	(150)
Pinus 1	Pine shoot	E/W 70 30	0.60–0.85	Oat coleoptile extension Wheat root extension	—	(64)
a	Wheat root	B/A/W	0.05–0.10	Oat coleoptile extension Wheat root extension	—	(116)

* The solvents employed were: W = Water, A = 0.880 Ammonium hydroxide, *i*P = *iso*propyl alcohol, H = *n*-Hexane, B = *n*-Butanol, E = Ethanol.
† The reagents employed were: E = Ehrlich, S = Salkowski, G = Gordon & Weber, N = Nitrous/Nitric acid.

in soil humic acid (62) needs further confirmatory evidence. Conversely, the conclusion on the basis of bioassay and R_F that IAA is present in Maryland Mammoth tobacco (101) has been shown to be doubtful (215, 216, 217). Likewise, the growth promotion in the bioassay by extracts from sycamore, after paper chromatography, was attributable to sugars rather than to indole auxins (149a) and similar non-hormonal growth promotion was reported with extracts from potato and lettuce (26), and banana (137).

The finding that an acidic precursor remaining in the water extract of cabbage after ether washing gave a neutral growth substance (25) was confirmed (91). The precursor was decomposed either by repeated chromatography in ammoniacal solvents or by heating at 100°C., and among the auxins produced was one which behaved like IAN (91). Analogous results were noted using ascorbigen and ammoniacal solvents which also gave auxins, one of which appeared in the IAN zone (107), although the fact that the precursor is active (91) and ascorbigen is inactive (161) in the oat cylinder test must not be overlooked. A similar hydrophilic complex from cabbage was also partially purified by countercurrent distribution, and its breakdown to IAN verified (156). The precursor that diffused from oat coleoptiles was also inactive but was transformed to an auxin either spontaneously or on standing at pH 3 [Raadts & Söding (162)].

Interconvertible auxins were first reported in extracts from tomato roots [Britton *et al.* (30)]. When chromatographed on paper in butanol/ammonia, active zones were found at the position of IAA (R_F 0.15), at R_F 0.5, and at the position of IAN (R_F 0.85); these were called *X*, *Y*, and *Z* respectively. It was shown that *X* and *Y* were interconvertible and that each could form *Z* on rechromatography. There was also some evidence that *Z* could form *X* and *Y*. The zones of growth promotion obtained on chromatograms of maize coleoptile or root extracts also behaved in a similar manner [Housley *et al.* (92)]. Likewise, the substances extracted from algae also exhibited chromatographic properties closely similar to those of *X*, *Y*, and *Z*. It was emphasized that conversion of one zone to another was never complete and there appeared to be an equilibrium between them [Bentley (16)].

The two auxins, *e* and *f*, from maize kernels were also reported to exhibit convertibility during chromatography. The substance *f* was partially converted to *g* whilst *e* was readily converted into *f* and *g*. All three substances gave a positive reaction with Ehrlich's reagent. The compound *f* was shown by chromatography to be tryptamine [Hemberg (86)]. Similarly, the four growth substances extracted from pea roots also exhibited spontaneous interconvertibility even when examined in neutral solvents [Audus & Gunning (9)].

The bulk of the auxin activity in extracts from Calimyrna figs was found to occur at the IAA position on the chromatogram (R_F 0.20 to 0.32 in *iso*-propanol-ammonia solvent), yet when the auxin was eluted from the paper with aqueous-ethanol and re-chromatographed, it was converted to an auxin with an R_F of 0.52 to 0.62 [Crane *et al.* (47)]. These results not only add to our knowledge of interconvertible auxins but also demonstrate again

that auxins detected at the IAA position on chromatograms should be subjected to further decisive experiments.

It has not been established whether any of these interconvertible auxins are indole compounds but it would appear that *Z* from algae (Table I) was probably an indole auxin (15, 16), as were the substances *e*, *f*, and *g* (Table I) from maize kernels (86).

Utilisation of Auxins

Metabolism studies of indole auxins in plant tissues (19, 59, 75, 113, 178) or in cell-free tissue extracts (200, 204) have provided information on auxin utilisation. For example, when a range of auxins was examined, namely IAN (19, 59, 178, 200, 204), γ-(indole-3)butyric acid (58, 59), ethyl indole-3-acetate (55), IAAld (19, 115), and IPyA (18, 49), IAA was detected by chromatography, thus showing that these compounds may function as precursors of IAA. However, when IAA and β-(indole-3)propionic were subjected to metabolism in various tissues (5, 13, 58, 59, 73) the evidence obtained by chromatography indicated that these were not precursors of more important unknown auxins. The possibility of N,N′-di-indolediacetic acid formation (90) has not been confirmed (13, 42). It would appear, therefore, that we can tentatively accept IAA and β-(indole-3)propionic as auxins that are active per se (58). The formation of indole-3-acetylaspartic acid from IAA metabolised in plant tissues (5, 13, 73, 109) could be regarded as a detoxification process since a search for this compound in plants has not shown that it occurs naturally. It is of interest that IAN inhibited its formation [Bennet-Clark & Wheeler (13)].

IAN was originally described as a naturally occurring plant growth hormone [Jones *et al.* (95)] and its greater activity than IAA in certain tests was emphasised (17, 20, 193) although this was also regarded as possibly due to its facile penetration into the tissues. The role of IAN as a precursor of IAA was subsequently shown in such tissues as oat (19, 204), barley (204), and wheat (59, 178) in which it was also highly active. Since, in other tissues such as maize, pea, lupin, tomato, and celery, IAN showed negligible or slight auxin activity [results that correlated with poor conversion to IAA as confirmed by chromatography (59, 117, 178, 204)], IAN was regarded as inactive per se (75). Most of the IAN found in members of the genus *Brassica* was located in the leaves (120), and reasons for thinking that IAN itself does not function as a hormone in cabbage (from which 2 mg/kg were extracted) have also been discussed (156).

It is now apparent that the activity of IAN is not always governed by its conversion to IAA. Thus, by comparing the action of IAA and IAN on sporelings of water-fern, it was found that IAN affected morphological development differently than did IAA. [Allsopp (2, 3)]. The most striking response to the nitrile was the marked elongation of both petiole and internode. These processes were accompanied by a considerable increase in cell number and were not explicable merely by cell extension. Similarly, a study of the inhibition of main axis growth and of alterations in lateral frequency in

excised tomato roots, induced by mixtures of IAN and IAA, supported the view that IAN affected root growth differently than IAA [Street *et al.* (195)].

Another contribution to this question was provided by a study of the occurrence of IAN, its conversion to IAA, and the auxin activity of IAN in tissues of 15 different species. Although IAN did not appear to undergo conversion to IAA in *Impatiens*, *Solanum*, and *Helianthus* spp., yet in these tissues it exhibited high auxin activity. It was concluded that IAN can cause cell elongation by two processes—either through hydrolysis to IAA or by virtue of its own activity [Libbert & Ballin (117)].

When IAN was hydrolysed enzymatically or subjected to metabolism in plant tissues, indole-3-acetamide was not detected (59, 178, 193, 204). Naturally occurring indole-3-acetamide has not been found in plants, but when IAA was incubated with plant tissues the amide was detected by chromatography [Good *et al.* (73)]. It has also been reported as an artifact formed during ammoniacal chromatography from indoleacetylglucosiduronic acid and other indole compounds [Jepson (93)].

The *modus operandi* of indole auxins which are active per se is a most important aspect of auxin utilisation. The comprehensive review on this subject by Galston & Purves (70) concluded that no one theory satisfactorily explained the mechanism of auxin action. Since then the chelation theory in particular has been subjected to serious criticisms (35, 51, 54).

Free and Bound Auxin

Free auxins have been delimited to those extractable in 2 hr. from plant tissue in ether at 0°C. in the dark, whilst the term "bound auxin" is used for the auxin liberated from the remaining indole compounds and complexes when subjected to enzymolysis, hydrolysis, or autolysis (31, 32, 86, 114, 143, 226).

Bound auxin apparently exists in the plant in various forms which include IAA-protein (69), tryptophan-protein (174), tryptophan, ascorbigen, and interconvertible auxins. All of these may liberate IAA when treated with hot alkali. At present it is not possible to calculate what fraction of the total IAA liberated by such treatment in bound-auxin studies (32, 114, 143, 226) is contributed from each of these forms.

Although the abundant quantity of bound auxin obtained from maize endosperm was thought to have little physiological importance [Yamaki & Nakamura (226)], the liberation of IAA by hot alkali from ascorbigen (160, 161) [considered to be important physiologically (10, 107, 110, 209)], the new cold alkali technique used by Bentley to examine certain interconvertible auxins (15, 16), and the active state of auxin found in wheat root [Fransson (65)], would appear to open up new lines whereby the generalisation of Yamaki & Nakamura can be subjected to further tests.

Synergism

The rooting of *Ageratum* petioles (163) and *Phaseolus* cuttings (78) induced by IAA was increased synergistically by the addition of indole, and

the rate of IAA oxidation by an enzyme preparation from etiolated pea seedlings was decreased in the presence of indole (164). However, phenylacetic acid also exerted a synergistic effect on the rooting capacity of IAA [van Raalte (163)] but did not protect IAA against the pea oxidase [van Raalte (164)]. Hence, it would seem that phenylacetic acid is not acting like indole, that is, as a substrate for the IAA-oxidase. The pea IAA-oxidase inhibitors, which are usually unsaturated acids (85, 187), also appear to act differently than phenylacetic acid. The auxin sparing actions of gibberellic acid (70), which has the structure of a cyclised phenylacetic acid derivative, may provide a clue to the mechanism by which phenylacetic acid is operating. Alternatively, the mechanism may be as suggested for the synergistic inhibition of main axis growth obtained with various combinations of IAA and IAN in excised tomato roots. The results indicated differences in the action of these two substances on separate components of the total elongation process [Street *et al.* (195)].

The synergistic action or the auxin-sparing effect of IAN in the presence of IAA was also obtained in slit pea stem tissue (19, 132, 141) but not in pea stem segments (132) or in oat coleoptile segments (19); yet, 5,7-dibromo-2-methylindole-3-acetic acid synergised with IAA in oat segments (63). Experiments to determine why IAN synergised with IAA in the slit pea test but not in the pea segment test provided evidence that was inconclusive but mostly contradicted the idea that IAN acted by sparing IAA from IAA-oxidase [Michel (132)].

Other classes of compounds are also known, including kinetin, that can potentiate IAA action in plant tissues. Thus, coumarin pretreatment for 3 hr. was synergistic for the elongation of sunflower hypocotyl and pea epicotyl segments with optimal concentrations of IAA [Neuman (136)], whilst thiourea synergised with IAA inhibition of root growth in germinating *Avena* and regeneration of shoot growth in defoliated *Avena* seedlings [Wynn-Parry (225)].

Although synergists frequently have structural features closely similar to the compound with which they synergise, and the synergism is often due to competition at a site of loss [Veldstra (212), Thimann (203)], it appears that other mechanisms may operate. Some of the above examples suggest this and also indicate that synergism of IAA by indole compounds is not only a more general phenomeon that hitherto realised, but is also an important aspect of auxin utilisation.

Ascorbigen

Following the observation that "ascorbigen," a complex which liberated ascorbic acid on hydrolysis, was extractable in ethanol from *Aegle*, various *Brassicae*, and *Phaselous* spp. (144), it was found that extraction of dried cabbage also yielded ascorbigen (182), which was non-dialysable and incompletely precipitated when metaphosphoric acid was used to denature the protein fraction (172).

Analysis showed that ascorbigen contained nitrogen and sulphur but

no phosphorus (71); but a proportion of ascorbic acid:phosphoric acid:protein of 1:1:1 was also reported (7). No sulphur was found in subsequent purified specimens and after ascorbigen was recognised as an indole derivative [Procházka *et al.* (157)], it was later characterised as an oxidation-resistant compound with ascorbic acid and 3-(indole-3′)prop-Δ^1-ene-1:2-diol moieties (Fig. 1) [Procházka (161)]. Ascorbigen was reported inactive in the oat cylinder test; but alkaline hydrolysis liberated IAA and indole-3-carboxylic acid whilst acid hydrolysis yielded ascorbic acid (161). Chromatography of ascorbigen in the ammoniacal solvents widely used in indole auxin research caused complete breakdown to give physiologically active compounds, including two that had R_F's similar to IAA and IAN (107). It was considered likely that the interconvertibility obtained by repeated chromatography of cabbage extracts in ammoniacal solvents [Housley & Bentley (91)] was explicable on this basis [Kutáček *et al.* (107)]. Unsuitable extraction procedures can also lead to no yield of ascorbigen from various *Brassicae* (107).

The ability of ascorbic acid to increase plant growth has been emphasised [Söding (186)], and striking interactions between IAA and ascorbic acid have been noted in oat (37, 173) and pea (205). Since ascorbigen is the only naturally occurring combined form of ascorbic acid so far isolated, it is of interest that the decrease in bound ascorbic acid in plants receiving extra molybdenum correlates with increased growth and increased nitrate utilisation [Kupke (106)].

The physiological importance of ascorbigen has been established by other recent studies. Thus, both ascorbigen and ascorbic acid could not be detected in Brussels sprout seeds until two days after sowing [Kutáček *et al.* (107)]. This significant increase in bound ascorbic acid content also occurred during the germination of legume seeds [Banerjee *et al.* (10)]. When the ascorbigen content of both white and red kohlrabi was followed during the first year of vegetative growth, a similar distribution throughout these two varieties was revealed. The young parts contained the highest levels and these fell rapidly with increasing age, thus showing a striking resemblance to the distribution and utilisation of naturally occurring growth substances (111). Similar results were obtained in a comprehensive study of radish varieties (*Raphanus* spp.). The tubers contained little ascorbigen compared with the leaves, most being obtained from young leaves; during the blossoming period, however, earlier differences between varieties almost disappeared [Valenta *et al.* (209)].

The ascorbigen in various organs of *Brassicae* not only paralleled the concentration of IAA present but was metabolised at a high rate, indicating that it was probably participating in growth (110). The highest levels were found in the tips (0.66 per cent), and the high levels in young leaves decreased with age, whereas the amounts of ascorbigen obtained from the buds increased with their development; it could not be found in the roots [Kutáček *et al.* (110)].

Ascorbigen was found in cauliflower curd and leaves (0.0005 and 0.002 per cent, respectively), together with quantities of IAN and indole-3-carboxylic acid [Procházka (158)]. Similarly, with the pure ascorbigen obtained from cabbage leaves the presence of IAN, indole-3-aldehyde, and indole-3-carboxylic acid was also proved by isolation and characterisation [Procházka & Šanda (159)]. Since it was considered that indole-3-carboxylic acid did not arise as an artifact (158), α-oxidation of IAN to indole-3-carboxylic acid in the plant can be inferred (56, 178).

Interconversions of Certain Indole Compounds in Plants

The conversion of ascorbigen to IAA within plant tissue has not been reported. Tryptophan, however, has long occupied a central position in schemes describing indole auxin biogenesis (21, 75, 76, 113, 121, 167), yet mechanisms of tryptophan synthesis in plants have hardly been explored. The pathway via anthranilic acid through indole-3-glycerol to indole, which condenses with L-serine, was established mostly by studies employing microorganisms (21, 129). However, rather than one pathway for the biosynthesis of indole derivatives, a network or lattice of pathways was favoured. The indole compounds were envisioned as being formed from amino acids via pathways involving either pyrrole derivatives or *o*-aminobenzene compounds. Implications of this "aryl lattice" were discussed and links with several important biochemical and physiological processes such as photosynthesis, respiration, differentiation, and growth regulation were emphasised [Bitancourt (21)].

When recent results concerning ascorbigen, IPyA, indole-3-pyruvaldehyde, and interconvertible auxins are put in perspective, the picture obtained (Fig. 1) suggests that an "Indole Cycle" (or Indole Cycles) may exist in plants. Some of the evidence that supports this new concept in auxinology will now be considered. The pathways depicted in Figure 1 briefly summarise conversions that have been demonstrated in a number of different plants; they will be discussed consecutively.

I. The tryptophan synthetase system from pea seedlings used indole and L-serine [Greenberg & Galston (79)]; likewise, tomato tissue and homogenates yielded not only tryptophan from these compounds but also IAA [Mudd & Zalik (134)].

II. The tryptophan content of pea and clover (213), oat (128), and tomato (206) plants remained fairly constant during vegetative growth, but in pea and clover it showed a significant increase before flowering and then afterwards decreased to its previous level. The suggestion was made that this tryptophan was converted to IAA at the time of flowering [Virtanen & Laine (213)].

III. Tryptophan biosynthesis via pyrroles and *o*-aminobenzenes has been suggested (21).

IV. Ripening ears of wheat synthesised tryptophan from IPyA (am-

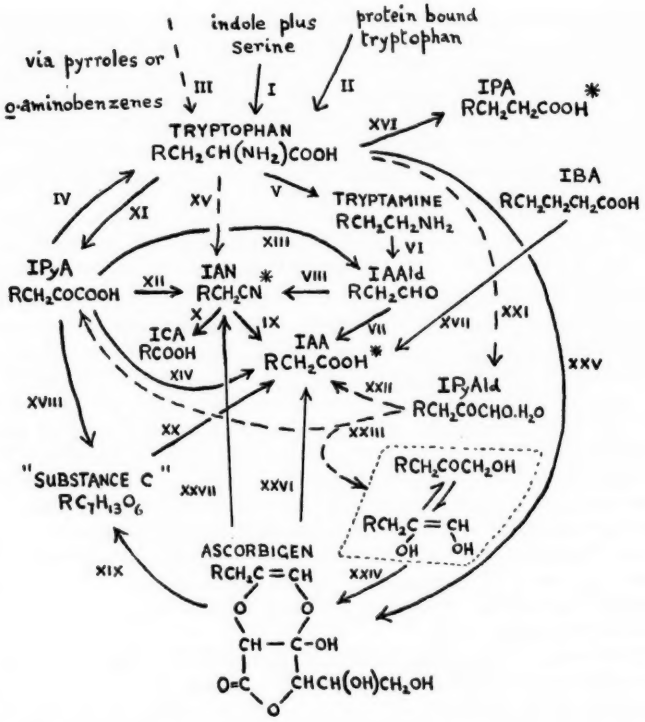

FIG. 1. Interconversions of certain indole compounds in plants.

In each formula, R represents the 3-substituted indole nucleus. All the compounds except the one (shown in its two tautomeric forms) enclosed by dotted lines, have been found in plants. Full line arrows show pathways for which evidence is presented; broken line arrows show suggested pathways.

* Tentatively accepted as an auxin active per se.

monium salt), and the nitrogen atom of the ammonium group entered the amino group of tryptophan. When potassium indole-3-pyruvate was used, a transamination involving other amino acids was apparently necessary [Kretovich & Polyanovskii (104)].

V. The pathway of tryptophan oxidation via tryptamine to IAA was proposed following the observation that tryptophan and tryptamine were slowly converted to auxin in oat tissue [Skoog (185)]; this conversion to IAA also occurs in carrot, flax, and radish (60), and probably in lentil (151) but not in mung bean (75). The formation of tryptamine from tryptophan has

been demonstrated in watermelon tissue [Dannenburg & Liverman (49)] and fungi (46).

VI. A purified amine oxidase from pea seedlings gave high yields of IAAld from tryptamine [Clarke & Mann (40)].

VII. IAA was formed from tryptophan via tryptamine and IAAld in pineapple [Gordon & Sanchez Nieva (76)] and maize (226). The oxidation of IAAld to IAA has been demonstrated in oat coleoptiles (112, 222).

VIII. The conversion of IAAld to IAN in plants was suggested (49), and experimental results indicate that in oat tissue the probable intermediate in this conversion is indole-3-acetaldoxime [Liverman & Dannenburg (123)].

IX. The hydrolysis of IAN to IAA (19, 59, 178, 200, 204) has been discussed and it was noted that indole-3-acetamide was not detected as an intermediate (49, 59, 123, 178, 193, 204).

X. The α-oxidation of IAN via indole-3-aldehyde to indole-3-carboxylic acid in pea and wheat tissues has been proved by isolation and characterisation of these products [Fawcett *et al.* (56)].

XI. Since the yield of IAA in *Rhizopus* cultures was observed to depend upon the tryptophan content of the media and the extent of aeration [Thimann (202)], pathways involving oxidative deamination of tryptophan to IPyA and oxidative decarboxylation of IPyA to IAA were suggested. Enzyme preparations from spinach leaf were found capable of converting tryptophan or IPyA to auxin, but incapable of so converting tryptamine (223). The level of free tryptophan was correlated with free IAA during the development of maize seedlings [Yamaki & Nakamura (226)] and strawberry fruits [Nitsch & Wetmore (140)], but in tobacco styles and ovaries a decrease in free tryptophan occurred when the auxin level was increasing [Lund (126)]. In watermelon tissue the main pathway for the conversion of tryptophan-2-^{14}C to IAA seemed to be through IPyA, based on the R_F and radioactivity of the IPyA relative to IAA [Dannenburg & Liverman (49)]; although this evidence was considered to be unconvincing [Kaper & Veldstra (97)], the pathway via IPyA was also found important in oat (123), pineapple (76), maize (226), and cabbage (108). The *in vitro* study of IAA formation from tryptophan by *Agrobacterum tumefaciens* (97) revealed a characteristic pattern of metabolites on the chromatogram, similar to that obtained from IPyA decomposition. By varying the incubation period it was found that IAA could be detected after 23 hr. but the evidence indicating IPyA formation appeared during the next 15 hr.

XII. IPyA may go via its oxime to IAN (191) but no evidence for this particular oximation in plants is known.

XIII. Aqueous solutions of IPyA decomposed to give IAAld (223), and an ultra-violet spectra study of IPyA in solution provided supporting evidence for this (175).

XIV. IPyA in solution decomposed to IAA and several other compounds (190, 223); likewise, evidence for IAA formation during chromatographic analysis on paper was obtained (18, 97, 192, 193).

XV. The production of radioactive IAN from ^{14}C-tryptophan in Savoy cabbage (108), watermelon (49), and oat tissue (123) could be via this alternative route, but since radioactive IPyA was also detected in these studies the other described pathways to IAN may be involved.

XVI. The de-amination of tryptophan to β-(indole-3)propionic acid has been demonstrated in microorganisms (43) but not in plants. This compound is much less active than IAA in certain tests, *e.g.*, in the oat curvature, oat segment, tomato epinasty, tomato rooting, and tomato parthenocarpy tests. This result is also observed in the pea segment test but in the slit pea curvature test IAA and β-(indole-3)propionic acid exhibit comparable activity. The likely reason for these differences has been discussed (59) and it also probably accounts for tryptophan being active in the pea segment test but not in the slit pea test [Simpson(184)].

XVII. γ-(Indole-3)butyric acid undergoes β-oxidation to IAA in plant tissues (6, 58, 59) and is more active than IAA in the slit pea test (59). Although easier penetration into the cells might partly account for this result, synergism or activity per se may also be important.

XVIII. Since enzyme preparations from cabbage juice failed to facilitate the biosynthesis of ascorbigen from IPyA and L-ascorbic acid it was suggested that the enzymes involved are unstable or bound to cell structures, and may need cofactors to operate *in vitro* (44). Incubation of IPyA and L-ascorbic acid for 48 hr. at 37°C. in phosphate buffer (pH 10.8) led to the production of "substance *C*" [Constantzas *et al.* (45)], which is known to occur in cabbage [Procházka & Sanda (159)].

XIX. In alkaline solution ascorbigen is rapidly converted to "substance *C*", a compound ($C_{15}H_{19}O_6N$) that has lost the carbonyl grouping known to exist in ascorbigen [Procházka *et al.* (161)].

XX. In hot alkaline solution "substance *C*" yields IAA (161).

XXI. The indole-3-pyruvaldehyde in cabbage may be formed by metabolism of tryptophan (156), but no evidence to support this view is given.

XXII. It is not known whether the high auxin activity of indole-3-pyruvaldehyde—which is more active than IAA in the oat curvature test (156)—is due to a facile conversion to IAA.

XXIII. Dismutation of aldehydes is a well known process, but neither IPyA nor (indole-3)acetylmethanol, the expected products, have yet been shown to arise from indole-3-pyruvaldehyde.

XXIV. The tautomeric occurrence of 3-(indole-3′)prop-Δ^1-ene-1:2-diol, which could condense with ascorbic acid to give ascorbigen, would appear likely. Moreover this diol and its tautomer, (indole-3)acetylmethanol, could account for two of the interconvertible auxins that were discussed earlier. "Substance *C*" was shown by analysis not to be this diol but was visualised as an ether derived from it (161).

XXV. The biogenesis of radioactive ascorbigen from tryptophan-3-^{14}C in Savoy cabbage [Kutáček *et al.* (108)] has confirmed the earlier work, which revealed ascorbigen formation after tryptophan was injected into the bulb

of kohlrabi (110). Further experiments will perhaps decide the relative importance of the pathways via IPyA and indole-3-pyruvaldehyde.

XXVI. Ascorbigen undergoes alkaline hydrolysis to give small yields of IAA (160, 161), and IAA is also formed in ammoniacal solvents during chromatography (107). Ascorbigen is also rapidly degraded after being infused into leaves of cabbage and kohlrabi, but IAA was not detected (110). Since ascorbigen is inactive in the oat coleoptile test (161) its conversion to IAA in this tissue would appear to be negligible throughout the duration of the test.

XXVII. The chromatography of ascorbigen in ammoniacal solvents led to the formation of an auxin with properties similar to those exhibited by IAN (107). The conversion of tryptophan in cabbage to IPyA, ascorbigen, and IAN (108) could also be along this pathway.

Thus, from a consideration of all these experimental facts it is concluded that the regulatory system (Fig. 1), which accounts for the balance of indole auxins found in various plants, has cyclic characteristics. It also appears probable that different cycles are predominant in different plants. The one instance of reversibility shown in Figure 1 is represented by pathways IV and XI. Since pathway XI from tryptophan to IPyA was demonstrated in spinach, maize, pineapple, oat, watermelon, and cabbage tissues whereas the pathway back to tryptophan was located in wheat tissue, it is still necessary for even this reversibility to be shown in one species.

Another striking feature of Figure 1 is the manner in which numerous pathways lead to IAA. Examples have already been noted, however, of a pathway becoming well defined in one species but appearing unimportant in others. Thus, conversion of IAN to IAA by pathway IX is well defined in *Triticum* but appears unimportant in *Impatiens*, *Solanum*, and *Helianthus spp.* [Libbert & Ballin (117)]. Similarly, from a range of species examined, tryptamine exhibited the least auxin activity and the minimum conversion to IAA in lucerne, oat, sugar-beet, and hemp, but the most auxin activity and the maximum conversion to IAA in carrot, flax, and radish [Ferenczy (60)]. Such results are also consistent with the view that different species have evolved their own specific pathways of IAA production.

Certain other routes and interconversions are less definitely established and indole-3-glycollic acid (80) has not been included in Figure 1, but useful hints to some of the as yet unknown pathways have been reported. For example, in excised wheat embryos the earlier and faster biosynthesis of ascorbic acid from sucrose catalysed by IAA is considered of significance in vernalisation and in the flowering of crop plants [Chinoy & Nanda (38)]. This may also mean that ascorbigen production is IAA-mediated. By directing attention to mechanisms and pathways of known and probable importance, the concept of "Indole Cycles" can aid the planning of research in plant physiology and biochemistry. It is still not possible to say whether the results obtained will remain academic or lead to practical measures enabling greater human control of plant growth.

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APPLIED ASPECTS OF THE GIBBERELLINS[1]

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Introduction

The history of the gibberellins and reports of their physiological activities have been capably reviewed (14, 15, 17, 168, 169, 170). Phinney & West have assembled the evidence (146) confirming the chemical structures for the various gibberellins. Some plant responses to gibberellin have been cataloged in terms of their actual or potential usefulness in crop production (35, 201). Appraisal of these benefits will necessarily depend on the accumulated experiences of competent investigators working with a wide range of environmental conditions. To date the number of beneficial applications of the gibberellins is limited. Many reports of growth responses are contradictory or inconclusive and further trials are needed before final conclusions are possible. Consequently the present review must be somewhat tentative in its evaluation of benefits to be derived from the use of gibberellin on economic plants. This review will be limited to a discussion of typical growth responses to the gibberellins in terms of how the plant's physiology has been modified, together with some comments on factors thought to be of importance in determining the response potential of intact plants.

Terminology

The gibberellins are defined (146) as substances possessing the same carbon skeleton as gibberellin A_3 (gibberellic acid), or one very closely related to it, and biologically active in stimulating cell division, cell elongation, or both, in plants. The term gibberellin(s) is used rather loosely in the literature as being synonymous with gibberellic acid. Only small amounts of the other gibberellins have been available for testing; so results discussed in this review, unless otherwise stated, refer only to gibberellic acid or its potassium salt.

Relative Effectiveness of the Gibberellins

The acids.—Thus far, nine distinct gibberellins have been isolated, six from the metabolic products of the fungus *Gibberella fujikuroi* (Saw.) Wr., A_1, A_2, A_3, A_4, A_7, A_9, and four from the immature seed of *Phaseolus multiflorus* Lam., A_1, A_5, A_6, A_8 (50, 119, 197). Gibberellin A_1 was also isolated from citrus and *P. vulgaris* L. (146, 197). The biological activity of the first four gibberellins, namely A_1, A_2, A_3, and A_4, has been characterized. Gibberellin A_3 was the most active in promoting epicotyl extension in bean,

[1] The survey of literature pertaining to this review was concluded September 20, 1960.

maize, morning glory, and *Chrysanthemum* (24, 33, 86), in reducing the time required for flower primordia to appear in lettuce, and in inducing parthenocarpic fruit growth in tomatoes (24). The remaining gibberellins were less effective in the following order: $A_1 \simeq A_4 > A_2$. When the concentrations of the less active gibberellins were increased, the differences among the four gibberellins were less apparent. When the four gibberellins were tested on extension of the epicotyl and the number of flower primordia formed on plants of *Ipomoea nil* (L.) Roth cv. Kidachi, a sequence ($A_3 > A_1 > A_4 > A_2$) similar to that found with most plants was observed (87). At a high dosage (100 p.p.m.), the sequence was reversed for the number of flowers, namely $A_2 > A_4 > A_1 > A_3$, suggesting that excessive stem extension reduced the number of flowers formed. Since A_2 was the least active on stem extension, it had little effect in reducing the number of flower primordia.

Several exceptions to this order have been observed. When compared on cucumber seedlings, A_4 was much more active than A_3 in promoting hypocotyl (74) and epicotyl growth (203), reversing the inhibition of stem growth by red light (74, 112), and inhibiting root extension (74). When applied in total darkness, gibberellin A_4 was 50 to 100 times more effective than A_1, A_2, and A_3 in promoting the dark germination of *Nicotiana tabacum* L. (78) and lettuce (181), as well as the expansion of dark-grown *Phaseolus* and *Raphanus* leaf discs (78). Gibberellins A_1, A_5, and A_6 were equally active on dwarf maize but A_8 was relatively inactive (119). Many other growth processes are undoubtedly controlled by specific forms of the gibberellins. With the almost ubiquitous occurrence and action of the gibberellins, many growth phenomena as yet uncontrollable may eventually be shown to be modified or capable of regulation by native or applied gibberellins. From the observations thus far, A_1 and A_3 showed similar effectiveness on various physiological phenomena related to epicotyl elongation. In contrast, A_4 accelerated expansion of leaf discs and initiation of seed germination. These two processes have been shown to be operative only when stem extension was limited or not involved. The response of various members of the Cucurbitaceae was the only exception observed. Thus far gibberellin A_2 has exhibited little physiological action under any circumstance.

The salts.—On a molar basis, ammonium, calcium, rubidium, copper, silver, lead, manganese, cobalt, potassium and sodium gibberellate, and gibberellic acid cyclohexyl amine salt were equally active on the growth of pea seedlings (124, 142). Potassium, sodium, and zinc gibberellate were as effective as the acid of A_3 in promoting hypocotyl extension of cucumber seedlings (74). Only the acid form promoted germination of *Lepidium* seeds (181) within a narrow range (2 to $2.5 \times 10^{-3} M$), suggesting control by a seed coat factor.

The acyls.—The acetyl, butyryl, benzoyl, and diacetyl derivatives of gibberellin A_3 were as active as the acid when applied to the roots or to the leaves of pea (124).

The esters.—Esterification of the hydroxyl group of gibberellin A_3 had no

effect on its biological activity (142), whereas esterification of the carboxyl group and acyl derivatives of esters resulted in compounds inactive when applied to leaves (124, 142, 176), to cucumber seedlings (74), and to parthenocarpic fruit set of tomato (160), but slightly active when applied to pea seedlings grown in water solutions containing the gibberellin (124). The effects of several *n*-alkyl esters of gibberellin A_3 on the germination of lettuce in darkness were compared (160). Although the methyl, ethyl, *n*-propyl, *n*-butyl, *n*-amyl, and *n*-heptyl gibberellates increased the percentage of lettuce seed that germinated (160), dilute solutions of ethanol, used to dissolve the relatively insoluble compounds in water, promoted germination (181). The observed activity may have resulted from the presence of ethanol and slow hydrolysis of the esters into the acid. The complete lack of activity shown by acyl esters may have been due to their low solubility.

Surface-active agents.—A limited number of reports demonstrated that non-ionic, polyoxy ethylene sorbitan monolaurate (Tween-20) increased the sensitivity of the response of mutant dwarf-1 of maize (131) and the epicotyl in the bean to low concentrations of gibberellin (27). Other types of surface-active agents were without effect. This area of research needs further study.

Effects of Gibberellins on Crop Yields

Application of gibberellin to seeds, soil, or growing plants for increasing crop yields has been generally disappointing. In comprehensive field trials by Morgan & Mees (126) with wheat, potatoes, turnips, carrots, peas, runner beans, lettuce, celery, black currants, kale, and maize, treatment with gibberellic acid failed to increase yields. Vegetative growth of most of the treated plants was stimulated but was not accompanied by an increase in yield. Grass was the only crop to show yield increases from applications of gibberellin, but this treatment had no advantage over fertilizer applications for increasing the growth except in spring or autumn when the action of fertilizers was slow (201).

Results at variance with those of Morgan & Mees (126) were obtained in Australia (159). Treatment of winter pasture land (grass and clover) with gibberellic acid significantly increased the total yield of dry matter. Equally important was the stimulation at times of normally slow growth.

Lawn grass.—An initial stimulation of blue grass (*Poa pratensis* L.), following the application of gibberellin at various times of the year, was reported (46, 105). With late fall and early spring application the increase was followed by less growth and no over-all gain resulted. Plots treated biweekly during the summer produced more grass than the controls by the end of the season, but had less ground cover the following spring. A single fall application likewise resulted in less spring cover.

Treating planting material of the grass *Zoysia* with gibberellin did not improve the rate of turf establishment (211).

Corn.—Field tests with corn in the United States showed (40) that gibberellin promoted the elongation of internodes but decreased the leaf area.

Stover weight and grain yield were sometimes increased when gibberellin was applied near pollination time but early applications repeated at weekly intervals decreased grain yields. Alder, Leben & Chichuk (1) observed a significant increase in plant height but obtained no statistically significant differences between weights of treated and untreated plants cut for silage, nor in the weight of mature ears.

Soybeans.—The response of soybeans to seed treatments with gibberellin was studied at eight locations from Winnipeg, Manitoba to Coahoma, Mississippi (82). Stands and yields were reduced by application of gibberellin to the seed at planting. Treated plants were taller in the seedling stage but shorter at maturity with greater losses from lodging. Maturity, oil, and protein contents of the seed were not affected consistently by the gibberellin treatment.

Grain sorghum.—Gibberellic acid as a seed treatment for grain sorghum significantly increased the rate of emergence and growth of young plants. However, final stands, height, lodging, maturity, and yield were unaffected. As a seed soak or in single or repeated dosages to young growing plants under field conditions gibberellin had no apparent effect on any characteristic measured (95).

Cotton.—Application of gibberellin to cotton (*Gossypium*) as sprays, dusts, and side dressings increased vegetative growth but this response was offset by reduction in boll size and later maturation of the crop (183). At maturity the increase in growth did not affect yields of plants grown in heavy soils but did slightly increase yields of plants growing in light soils. The increased growth resulting from applications of gibberellin did not increase yields of plants growing under favorable conditions.

Direct applications of gibberellin into the corollas of one-day-old bolls reduced abscission. The maximum retention approached 100 per cent when natural retention was 30 per cent or more. About 50 per cent retention was observed during periods of low boll set—when only 10 per cent were being retained on untreated plants (184). Since vegetative growth, flower production, and fruit development of cotton occurred simultaneously over a relatively long period, gibberellin apparently promoted extension and modified the subsequent flowering. Gibberellin promoted retention of the bolls when applied directly but was not translocated to the vegetative shoots.

Coffee.—Alvim (3) reported on the use of gibberellin to induce simultaneous flowering and uniform fruit ripening in coffee (*Coffea arabica* L.). When the soil water supply was adequate, 60 to 70 per cent of the flower buds opened within 9 to 10 days after spraying with a concentration of 10 to 20 p.p.m. Fruit set was increased 400 to 700 per cent. When soil water was limited or the flower buds were small, higher concentrations of gibberellin—50 to 100 p.p.m.—were needed. Uniform ripening of fruits decreased the number of pickings and increased yields.

Sugar cane.—Treating seed pieces of sugar cane (*Saccharum officinarum* L.) with gibberellin initially increased the height of the plants, but at harvest

there was no significant difference in height. Repeated applications of gibberellin for a six-month growth period produced plants 30 inches taller than untreated plants with 10 inches greater length of millable stalks. The treatments did not affect the ultimate yields of total solids, sucrose, or juice purity (43).

Sugar beets, hops, and tobacco.—Gibberellin applications on sugar beet plants tended to increase the yield of beets but reduced their sugar content (144). Similar results were obtained in the production of hops (130). A spray application of 12.5 p.p.m. of gibberellin accelerated ripening of the cones by 10 days and increased yield by 39 per cent but reduced the content of the constituent of most interest, α resin, from 10.16 per cent in the untreated to 1.8 per cent in those which had received the gibberellin. Similarly, a reduction in nicotine content of tobacco following application of gibberellic acid to the plants was noted by Burk & Tso (30) and Parups (141).

Grapes.—Applications of gibberellin to seedless varieties of *Vitis vinifera* L. induced a higher percentage set of very large berries of Black Corinth and of large, elongated berries of Thompson Seedless (167, 188). Little or no translocation from treated to untreated berries was observed. Treatment of the leaves was much less effective than applications to the flower clusters. The time of treatment in relation to the effectiveness was often more important than the concentration of gibberellin. A prebloom spray, applied to Thompson Seedless when the clusters were 3 to 5 inches long, increased the length of peduncle, pedicel, and lateral shoots (190). Many undeveloped berries were formed and a reduction of the weight of fruit per vine resulted. Treatment from the time that most of the calyptras had fallen until the berries were not more than 4 to 5 mm. in diameter resulted in maximum berry size. Spraying later produced progressively smaller berries. The percentage of total soluble solids in the juice was reduced by most treatments of the vines with gibberellin (191). When level of crop was eliminated as a factor by hand-thinning procedures, applications of gibberellin, even with maximum berry enlargement, had no effect on the total soluble solids (192). The raisins and currants produced from berries treated with gibberellin were of inferior quality (99).

Applications of gibberellin to seeded varieties of *Vitis vinifera* at bloom stages, the best time to treat seedless varieties, resulted in development of many berries which remained small, hard, and green. Prebloom sprays increased the length of the peduncle and pedicels, reduced the possible incidence of rot, hastened flowering and coloration in the varieties Zinfandel and Carigname (191), and reduced yield. Gibberellin had a physiological effect on seeded berries but only to a limited extent. It compensated for the lack of seed, but once the berry had a sufficient number of seed, gibberellin had no further effect (103). As demonstrated by Coombe (44), seedless varieties produced naturally occurring gibberellins whereas none were found in several seeded varieties.

Applications of gibberellin to seedless varieties of *Vitis labrusca* L. in-

creased berry and cluster sizes in seedless Concord and Interlaken (161), but slight increases in number and weight of berries of seeded varieties were observed (22). Berries of a seeded variety, Delaware, matured 15 to 20 days earlier than untreated berries when the clusters were sprayed 10 or 20 days before full bloom. All the berries in the treated clusters were seedless and larger than untreated seeded berries (139).

Other fruits.—Evidence is accumulating that the gibberellins can affect fruit setting and development in genera other than *Vitis* (70, 205). Thus Luckwill (117, 118) reported that gibberellin stimulated parthenocarpic development in some *Malus* and *Pyrus* cultivars. Vegetative shoot growth was unaffected. Griggs & Iwakiri (68) reported extensive trials with gibberellin applied to Bartlett pear trees at concentrations from 10 through 500 p.p.m. in September and at four separate times during the following spring. The study determined the effects of these sprays on the bloom period, fruit set, size, shape, color, pressure test, soluble solids, stem length, production of late bloom and abnormal fruit, vegetative growth, and flower bud formation. September sprays were ineffective but applications at blooming generally increased parthenocarpic set, produced longer fruit and stems, and many freak calyx end protrusions. Higher concentrations reduced flower bud formation.

Crane, Primer & Campbell induced parthenocarpy in some species of *Prunus* with aqueous sprays of gibberellin applied either at full bloom or at full bloom with a second application made 8 days later (49). Failure to induce parthenocarpy in the cherry and plum was thought to be due to the unfavorable environment produced by the muslin bags used to prevent cross-pollination. Two applications of gibberellin brought about parthenocarpic fruit sets of 11.8 per cent in the self-incompatible Jordonola almond and 15.4 per cent in the Royal apricot. Fruit set in the peach was increased by all concentrations of gibberellin applied (50, 250, and 500 p.p.m.) and ranged from 63 to 73 per cent as compared with 42 per cent of the open-pollinated blossoms. Parthenocarpic fruits of all species produced with 500 p.p.m. of gibberellin were nearly as large as open-pollinated control fruits, though somewhat different in shape. They ripened from several to as much as 10 days before the open-pollinated fruits. Stimulated vegetative shoot growth from spurs, delayed vegetative bud development, and prevention of flower bud formation, as a result of gibberellin treatment, occurred with all species except the peach where the buds appeared unaffected.

The flowering and fruiting of bearing peach and cherry trees in 1958 was not affected by applications of gibberellin made during full bloom in 1957 (83). A post-bloom spray of gibberellin on bearing cherry and peach trees resulted in partial inhibition of flowering in cherry trees and complete inhibition of flowering of peach trees the following year. Similar spray treatments on bearing apple trees had no effect on flowering or vegetative growth.

Hull & Lewis (83) observed that one-year-old greenhouse-grown cherry trees that were sprayed with gibberellin after they had formed terminal buds

produced a second flush of growth. As a result, treated trees produced significantly greater trunk diameters and greater total dry weight of the tops than the controls. Repeat applications of gibberellin were not beneficial. Some indication of the degree of species specificity in response is shown by the work of Powell, Cain & Lamb (150) who noted little or no benefit from applications of gibberellin to young seedlings of apple and pear.

Fruits of Thompson Improved navel orange just beginning to lose green color were dipped in gibberellin. The control fruits were more orange in color when harvested. The treated ones contained a higher percentage of juice and more ascorbic acid and had a smoother and thinner rind than the controls (42). In Japan, treatments applied at flowering improved fruit set but reduced the size and changed the shape of the fruit (138).

Smith (164) demonstrated that three autumn applications of gibberellin (1 or 2 weeks apart) increased the volume of strawberry fruit harvested by 30 per cent in the first three harvests as compared with the unsprayed plants. The total yields from eight harvests showed no significant difference between unsprayed plants and those sprayed with 10 p.p.m. gibberellin. Dosages higher than 10 p.p.m. reduced yields.

Vegetables.—Many vegetable crops respond to gibberellin by an increased rate of stem extension or by earlier flowering (200). Until further studies are made, most of these responses remain more of academic interest than of economic value. The behavior of celery is an example. Increases in petiole length and fresh weight of field-grown celery following treatment with gibberellin were reported by Bukovac & Wittwer (23). Further tests confirmed these results (26). However, the authors reported certain deleterious effects including a shorter harvest period, seedstalk formation, and rapid breakdown in storage that appeared to be directly related to treatment with gibberellin. These undesirable effects may outweigh any benefits derived from an increase in petiole length or fresh weight. Better results were obtained when temperatures were low during maturation (177).

Harrington used the seedstalk-promoting tendency of gibberellin to prevent heading, induce bolting, and increase seed yield of tight-heading lettuce (76). When gibberellin was sprayed on lettuce plants at the 4- and 8-leaf stages of growth in amounts of 3 and 10 p.p.m., the seed crop matured about two weeks earlier and the yield was significantly increased over that of the controls. As used, the gibberellin substituted for deheading, which is said to cost four times as much and to be the greatest expense in growing this type of lettuce. The gibberellin treatment had no effect on germination of the resulting seed or growth of the subsequent crop (25).

Wittwer and colleagues (205) demonstrated that gibberellin induced fruit setting in the tomato, but growth was inferior to that produced by the indole auxins, the phenoxy or naphthoxy acids, or normal pollination (199). Synergism between gibberellin and indoleacetic acid in promoting tomato fruit growth was noted by Luckwill (116) but not by Gustafson (70). Wittwer & Tolbert (206) have extended these results to show that addition of 2-

chloroethyl trimethylammonium chloride to the gibberellin and indoleacetic acid resulted in growth of tomato ovaries significantly greater than that produced either by gibberellin or by auxin alone or in combination. It is probable that further progress in applying the gibberellins for economic advantage will be made as knowledge of synergism with other growth-regulating materials is acquired and applied.

Flowers.—The dominant effects of gibberellin on plants cultivated for their flowers are: (*a*) replacement or partial substitution for their cold requirement (10, 172), (*b*) acceleration of flowering (36, 90, 108), and (*c*) apparent enlargement of the inflorescence (51, 109). The effect on influorescence size of *Pelargonium* was attributable to increases in pedicel length as well as in petal size. The lasting quality of the inflorescence of some varieties was enhanced. Replacement of the cold requirement for the forcing of azaleas required massive applications of gibberellin (10), while the effects on certain other flowers were achieved with very small amounts and overtreatment was ruinous (109, 173).

Trees.—Marth, Audia & Mitchell (120) reported that several deciduous species gave spectacular results but that growth of few conifers was affected by applications of gibberellin. Gibberellin significantly increased growth when applied to young stems of eastern cottonwood (*Populus deltoides* Bartr.), American sycamore (*Platanus occidentalis* L.), yellow poplar (*Liriodendron tulipfera* L.), sweet gum (*Liquidambar styraciflua* L.), cherry bark oak (*Quercus falcata* Michx. var. *pagodaefolia* Ell.), willow oak (*Quercus phellos* L.), and southern red oak (*Quercus falcata* Michx.) (133). Comparatively small effects were observed on the growth of conifers (120, 198). An exception was gibberellin-treated *Juniperus media* van Melle var. *pfitzeriana* (Beissn.) van Melle (120), which grew much more than untreated plants. Growth of *Taxus cuspidata* Sieb & Zucc. (122) and *Cupressus arizonica* Greene (133) was retarded or damaged by treatment with gibberellin. Injection of gibberellin directly into the xylem had no effect on the growth of poplar (73) but increased the stem weight of *Eucalyptus* spp. at the expense of leaf and root weights (158). The total weight was not affected.

Discounting time of treatment and concentrations of gibberellin the difference between response or non-response of trees depended upon their inherent growth characteristics. Trees of such genera as *Platanus*, *Populus*, and *Liriodendron* grew continuously under long days, but stopped with daylengths less than 12 hr. Gibberellin promoted extension by increasing the length of the internode rather than increasing the rate at which nodes were formed. Species of *Quercus* and *Pinus* also responded to long days by making several flushes of growth interspersed with periods of dormancy but stopped growing on short days (136). *Quercus phellos* L. and *Pinus taeda* L. responded to applications of gibberellin but many other species in these genera grew without an apparent response to gibberellin.

Treatment of seedlings with concentrated or frequent applications of gibberellin reduced the root growth of yellow birch (*Betula alleghaniensis*

Britton) (104), reduced survival of longleaf pine (*Pinus palustris* Mill.) seedlings (98), and slightly increased the growth of loblolly pine (*Pinus taeda* L.) (8).

Although the vegetative growth of conifers, with certain exceptions, appeared to be unresponsive, many species in the families Taxodiaceae and Cupressaceae (but not in Pinaceae) flowered earlier when treated with gibberellin (97). Treatment with gibberellin during July stimulated flower-bud differentiation on seedlings as well as on older plants of *Cryptomeria japonica* (L.f.) D.Don (96). When the induced flowers were pollinated artifically, most of them fruited. The size of the cones was smaller but contained the same number of seeds as those from untreated plants. Massive applications of gibberellin in lanolin to the terminal wounds on the cuttings hastened pollen shed from *Acer*, *Betula*, and *Prunus* by 9, 16, and 9 days respectively. Although anthesis was unaffected, the size of the flower parts increased in *Populus* (102).

Effect of Gibberellin on Specific Physiological Stimuli and Responses

Assimilation and translocation.—Since growth promotion involves expenditure of energy, some enhancement of CO_2 fixation might be anticipated as a consequence of treatment with gibberellin. However, Haber & Tolbert (71) reported that gibberellic acid did not increase the rate of CO_2 fixation per unit of leaf tissue. Their conclusions were based on tests with detached leaves of barley, oats, and peas set in vials containing varying concentrations of gibberellic acid for 2 hr. and then exposed to $^{14}CO_2$ in sealed containers for 30 min. Total fixed ^{14}C was counted in extracts of the leaves and found to be unchanged by the exposure to gibberellic acid.

Alvim (4) found that both the net assimilation rate and relative growth rate of kidney beans grown in solution culture were increased by spraying the plants with gibberellic acid. The increase in dry weight occurred in the stems and was thought to result from more rapid translocation of photosynthates from the leaves to the stems. Thus Alvim concluded that results obtained with detached leaves are not comparable to those obtained with intact plants. Coulombe & Paquin observed (48) a rapid increase in the respiration, photosynthesis, and transpiration rates of tomato plants in 5 or 6 hr. after spraying with gibberellin. Pre-treatment of Red Kidney beans with gibberellic acid 48 hr. before 2,4-D application to a single primary leaf increased the amount of 2,4-D found in the untreated areas after 24 hr., apparently because of increased translocation initiated by the gibberellin [Ashton (5)]. Linck & Sudia (107) observed that treatment of bean plants with 1 p.p.m. of gibberellic acid resulted in more accumulation of ^{32}P than in untreated plants.

Stuart & Cathey (174) obtained results similar to those of Alvim (4) with chrysanthemums grown in sub-irrigation nutriculture. Percentages of total mineral elements and nitrogen were highest in the stems of control plants, but the total amounts per stem progressively increased with increas-

ing concentration of applied gibberellin. Leaves and stems produced after gibberellin treatment contained higher percentages and total amounts of soluble carbohydrates than were present in similar untreated plants. Suboptimal amounts of nutrient nitrogen limited the total amount of stem growth but increased the percentage of stem elongation of gibberellin-treated plants. The presence of about four times as great a percentage amount of soluble carbohydrates in the nutrient-deficient stems as in those not restricted by deficiencies, may have contributed to the gibberellin-induced growth response.

Van den Ende & Koorneef measured significantly increased osmotic pressures in tops and leaflets of tomato plants that were sprayed with potassium gibberellate, and they associated this property with a lower yield of fruit than was obtained from unsprayed plants (182). Others have noted increases in weight of gibberellin-treated plants over those that were untreated (58, 82, 84, 120, 173, 201). At 0 and 1.5 atmospheres osmotic pressure, Nieman & Bernstein (135) observed that gibberellin overcame the decreased growth rate by increasing stem length, fresh and dry weight yield of fruit, and leaf area of dwarf Red Kidney beans. At 3.0 and 4.5 atmospheres, however, gibberellin increased stem length only. Increased rates of water use per unit leaf area were observed with the gibberellin-treated plants. A larger leaf area on the treated plants, better exposure of the leaves to the available light because of stem extension, and possible photosynthetic activity by the stems may help to account for the increased growth of the treated plants.

The generally observed temporary paling of leaves on gibberellin-treated plants is not directly due to the treatment but to pigment dilution arising from failure of chlorophyll synthesis to keep pace with increased cell expansion (207). Subsequent small decreases in chlorophyll content of treated plants were thought to be due to malnutrition. Thus it appears that no simple explanation of relation of action of gibberellin in photosynthesis to subsequent plant development is at hand.

Respiration and enzyme activity.—Gibberellin is reported to be effective in stimulating the respiration of germinating pea seeds (175). In fact, Nielsen & Bergqvist (134) proposed that the increased rate of respiration of barley grain as a result of treatment with gibberellin might serve as an assay method for gibberellin.

Some 20 years ago Hayashi (80) observed that gibberellin stimulated the germination of barley and increased its content of amylase. The significance of this finding and its application in the brewing industry seem to have been overlooked until Munekata & Kato (127) called attention to the higher α-amylase activity and effectiveness of malt following gibberellin treatment of the barley. These findings were confirmed elsewhere (148, 157) and at present gibberellin is finding wide acceptance in the malting industry (199). Apparently the benefits of gibberellin treatment derive from an increased percentage of germination as well as enhanced enzyme content of the malt.

The influence of gibberellin on other enzyme systems has been studied,

but the results do not permit ready interpretation in terms of physiological activity (81, 196).

In a series of remarkably clear-cut experiments, Paleg (140) demonstrated that the addition of gibberellin to barley endosperm increased the liberation of reducing sugars. Pre-treatment of endosperm with gibberellin increased subsequent amylase assay activity. The presence of gibberellin in the assay had little effect on amylase activity when the endosperm was not pretreated. This suggested that the effect of gibberellin was to increase the amount of enzyme rather than its activity.

Nodulation.—Applications of gibberellin to young plants of *Phaseolus vulgaris* L. (64, 179), *Medicago sativa* L., and *Vicia villosa* Roth., decreased nodulation but had little or no influence on nodule formation in older plants (123). With *Trifolium repens* L. (60) gibberellin had no effect on the growth of *Rhizobium trifolii* either in aseptic agar or in soil culture, or on the growth of the nodule forming organism. Inoculation of the gibberellin-producing organism *Gibberella fujikuroi* (Saw.) Wr. into the soil in which plants of *Pinus sylvestris* L. were growing decreased the vigor and the height of the plants (106). In podsols at pH 5, there was a shift in the proportion of the various root-associations affecting the plant. In untreated soil, the normal and pseudo-mycorrhizae were equally divided. In soil with *Gibberella* added, the pseudo-mycorrhizae were stimulated at the cost of the normal-mycorrhizae with a widespread increase in the parasitic association of the *Pinus* roots with *Rhizoctonia sylvestris.* Plants that were 8 months old showed a less pronounced contrast between mycorrhizal and non-mycorrhizal associations. The effect of gibberellin on the soil flora apparently depended upon the age of the plant and the particular genus involved.

Formulations with fertilizers and plant protectants.—Gibberellin could apparently be used with fungicides, bactericides (7,155), and fertilizers (137, 155) without any interference with the control of diseases and mineral nutrition. Further, these compounds did not alter the growth-promoting properties of gibberellin. When mixed with materials such as fungicides, antibiotics, and calcium salts, or when applied alone, gibberellin reduced the losses from the seedling disease complex of cotton (155).

Breeding.—Chandler (38) observed that gibberellin stimulated germination and growth of pollens from some plant species while it was inactive or deleterious to the growth of pollen of others (193). Potassium gibberellate applied to a relatively self-incompatible cultivar of *Lilium longiflorum* Thunb. improved seed set only slightly and was inferior to naphthalene acetamide (57). Combining it with the latter growth regulator produced variable results depending upon the time and method of application. Somewhat similar results were obtained on parthenocarpic fruit setting of three rose species (91, 151).

Gibberellin was reported as effectively inducing male sterility in two maize inbreds (132). The range of activity of gibberellin is shown by the work of Peterson & Anhder (143) and Galun (65), who were able to induce

staminate flowers at will by foliar applications of gibberellin to gynoecious strains of *Cucumis*.

Dwarfism.—One of the outstanding characteristics of the gibberellins is their ability to reverse genetic dwarfism (16, 145, 201). Dwarf segregants of *Tephrosia vogelii* Hook. f. were completely dependent upon periodic applications of gibberellin for normal growth (89). Similar results were reported for a genetic dwarf of *Lolium perenne* L. (45). Application of gibberellin to genetic-dwarf and normal tomato seedlings significantly increased stem elongation of both (166). However, dwarfness of some wheat varieties was not corrected by gibberellin and standard varieties were stimulated more than short varieties by injections of gibberellin (2, 208). Normal varieties of barley treated with gibberellin one week prior to meiosis responded as strongly as did the most responsive erectoid mutants (171). As found with single-gene dwarf mutants of *Zea mays* L., certain mutants did not respond (145, 171) to applications of gibberellin. Dwarfism was not due in all cases to a lack of production of gibberellin.

Dormancy.—Applications of gibberellin to dormant seeds promoted germination in an otherwise unfavorable environment. The germination of light-requiring seeds such as lettuce (85, 93, 113), *Parthenium argentatum* (guayule) (75), and *Lepidium virginicum* (181), occurred in total darkness with optimum concentrations of gibberellin. Concentrations below that stimulating dark germination increased the sensitivity of the seeds to a limiting exposure to light. A brief exposure to far-red radiation had only a limited inhibitory effect on gibberellin-promoted light-sensitive seeds. Fully promoted gibberellin-treated seeds were prevented from germinating when placed under continuous far-red illumination immediately after promotion treatment. Other light-requiring seed such as pine (*Pinus densiflora* Sieb. & Zucc.) (66) and Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco (154) did not respond to applications of gibberellin. Although the rate of germination was increased, total germination percentage was unaltered.

Lettuce seed was most sensitive both to applications of gibberellin and to red light after about $1\frac{1}{4}$ hr. of imbibition in darkness. This observation suggested that gibberellin acted in the embryonic hypocotyl as red light did. Yamaki *et al.* (209) reported that substances that induced germination of tobacco seed in darkness diffused out from bean leaf discs which had been illuminated with red light and that one of the substances had the same R_F value as the gibberellin. Ikuma & Thimann (85) were unable to demonstrate that even a trace of gibberellin A_3 or A_5 as assayed on dwarf maize was produced by light treatment of lettuce seed. Gibberellin A_4 was much more active than A_3 in promoting germination of lettuce (181) and tobacco seed (78), and expansion of bean leaf discs (78). The bioassay used by Ikuma & Thimann (85) would not have detected the presence of gibberellin A_4 in the acetone extracts since the dwarf maize used was responsive only to gibberellin A_1, A_3, and A_5. It has not yet been possible to determine if light induces germination of lettuce seed by liberating a gibberellin in the hypocotyl.

The germination of cold-requiring seed occurred when gibberellin was applied to partially cold-treated seeds such as sweet cherry (61) and peach (53), but gibberellin was inactive on unchilled seeds of peach and *Rhodotypos* (59, 123). Seeds of *Avena fatua* L. have been shown to exhibit embryo and pericarp dormancy. Stems of the plants were cut when the seeds were in the milk stage, allowed to take up potassium gibberellate from solution, and then placed in water. The freshly harvested seeds from these plants germinated completely within 10 days when the caryopsis was removed (9). Only 50 per cent of similar seeds with the caryopsis undisturbed germinated. The seeds from untreated plants were fully dormant. Gibberellin increased the number of dormant wild oat seeds germinating when a wetting agent was incorporated into the solution (67). The former chemical became more effective with increasing age of the seed.

Gibberellin increased either the rate, (63, 111, 180) or the total number, or both, of seeds germinating (61), but apparently did not replace all the cold requirements. The after effects were often undesirable, as the post-emergence losses were great (6, 12). Peach seedlings developed chlorotic leaves (59) and excessively elongate stems with narrow twisted leaves. Cherry seeds promoted to germination with gibberellin eventually set terminal buds and rosetted. Gibberellin treatments of cold-requiring seeds were sufficient to hasten the growth processes but did not persist long enough to maintain the plants in normal growth. A combination of soaking cherry seeds in a gibberellin solution and spraying every three weeks maintained growth. Presence of gibberellin during the onset of heat dormancy prevented its full development (147). The dosages of gibberellin necessary to overcome dormancy were extremely critical; concentrations too high inhibited growth while those too low were without effect (129). Seed coats of *Trollius* and *Geranium sylvaticum* L. pretreated with solutions of sulfuric acid were rendered permeable to penetration by gibberellin (94). Gibberellin or sulfuric acid alone were ineffective but in combination were highly effective on *Geranium* seeds. Toole & Cathey (181) observed that the germination of lettuce seeds was promoted equally well by the acid or potassium salt of gibberellin. However, *Lepidium* seeds required a higher and very restricted range of gibberellin concentrations for promotion of germination (2 to $2.5 \times 10^{-3} M$) and only the acid was active. The difference between the two kinds of seeds was due to the harder seed coat of *Lepidium*, which became mucilaginous on wetting. The potassium salt of gibberellin, even in concentrated solutions, was ineffective on the germination of *Lepidium*. Since it was necessary to alter the seed coat of some seeds with acid to allow gibberellin to penetrate, the embryos of many seeds reported thus far to be unresponsive may never actually have been treated with gibberellin. Gibberellic acid in a concentrated solution acted both as the penetrator and the stimulator of dormant *Lepidium* seed.

Gibberellin extended the range of temperature at which germination of bracted plantain (52), wild oat (67), and lettuce (181) could occur. It also promoted more uniform and earlier emergence of sweet cherry seeds in cool

soils, even of seeds after-ripened for six months (61). Similar growth responses of a variety of other crops have also been reported (92, 125); these depended on temperature and the depth of planting. Cotton seed planted 1½ inches deep exhibited an increased rate of emergence whereas seeds planted 2½ inches deep produced curled hypocotyls which were broken on emergence and ones planted ½ inch deep were unaffected. Treatments often overstimulated growth, increased the tendency to lodge, and reduced the vigor of the subsequent growth (12).

Foliar applications of gibberellin 1, 2, and 4 weeks before harvest resulted in visible sprouting on the developing tubers of white potatoes (110). Although the application of gibberellin was distant to the area of response, the onset of dormancy was prevented and growth was observed. However, treatment with gibberellin was not immediately active in promoting growth of all varieties of potatoes. Doorenbos (54) showed that gibberellin accelerated elongation of shoots, but did not reduce the length of the dormant period; the relative order of varieties as to time of sprouting was not changed.

Cessation of growth, the formation of buds, and the loss of leaves are the typical signs of the onset of dormancy. Weekly applications of gibberellin to vegetative plants delayed the onset of dormancy in *Acer pseudoplantanus* L., *Betula pendula* Roth., *Fraxinus excelsior* L., and *Liriodendron tulipifera* L. (19). Other tree species such as *Acer rubrum* L., *Fagus sylvatica* L., and *Quercus robur* L. exhibited no change in the development of autumn color and leaf fall. The following spring, bud break of *Acer pseudoplatanus* L., *Betula pendula* Roth, *Fraxinus excelsior* L., and *Sorbus aucuparia* L. (20), and *Vitis vinifera* L., (189) was delayed 1 to 3 weeks. Although plants of *Fagus sylvatica* L. did not respond to treatment in the fall, bud break was delayed several weeks the following spring (20).

The cause of cessation of growth of many woody plants has been shown to be due to the change in photoperiod (186). Application of massive amounts of gibberellin 2 to 4 weeks after the start of 8-hour photoperiods promoted growth of *Pinus elliottii* Engelm. (11). A single foliar spray of 50 p.p.m. applied to *Weigela* on 9-hour photoperiods increased the internode extension and the number of nodes. On 18-hour photoperiods, it did not alter internode formation but increased internode extension (21). Several woody plant species showed no response to applications of gibberellin on 8-hour photoperiods (136).

Some woody plant species without a photoperiod requirement for growth develop a block to growth which can be overcome only by exposure of the plant to low temperature. Applications of gibberellin to fully dormant *Aralia cordata* Thunb. (88) and *Hydrangea macrophylla* (Thunb.) Ser. (172) promoted stem extension and accelerated flowering. The dosages of gibberellin necessary to initiate growth of non-chilled dormant hydrangeas caused chlorosis of the new foliage and die-back of some of the growing points, and modified the inflorescence unless the leaves on the dormant plants were removed before treatment. Dormancy was by-passed and flowering of vegeta-

tive hydrangea plants was achieved by repeated treatment with gibberellin and indoleacetic acid (172). Plants of hydrangea (172) and peach (185), which had received part of their dormancy-breaking cold requirement, grew more rapidly when sprayed with gibberellin. The amount of gibberellin required to promote growth of partially chilled plants was much lower than that necesssary to overcome the dormancy of non-chilled plants, and high dosages applied to partially chilled plants over-stimulated growth. Since over-treatments of post-bloom trees partially inhibited flowering of cherry and completely inhibited that of peach the following year (83), the timing of gibberellin applications would appear to be extremely critical. Apple trees did not respond to treatments of gibberellin (83, 185). Applications of gibberellin did not always end dormancy. Spraying trees of *Taxodium distichum* (L.) Rich. induced a much earlier dormancy than was observed on untreated trees (20). Soaking freshly harvested aerial tubers of *Begonia evansiana* Andr. in solutions of gibberellin induced or prolonged dormancy. Weaver (189) observed similar effects with seedless grape varieties. The gibberellin-induced dormancy was broken by exposure to low temperature (129).

Vernalization.—Many publications indicate that applications of gibberellin to growing plants can replace, nearly quantitatively, effects either of vernalization or of long photoperiods in hastening flowering of rosette plants (55, 77, 202). Frequently repeated applications of gibberellin to vegetative plants caused seedstalk elongation and promotion of flowering. The final heights of gibberellin-treated, unvernalized endive plants grown on short photoperiods were twice as great as those of untreated, vernalized plants grown on long photoperiods. The former flowered as early as the latter and produced a similar number of leaves (77, 153). Although the pollen could be stained pink with acetocarmine, suggesting that it was viable, no seed developed on the plants until treatments with gibberellin were discontinued. The flowers that were formed, subsequently developed normal seed. A combination of seed treatment with gibberellin during vernalization caused a flowering response similar to vernalization followed by repeated foliar applications of gibberellin.

Most of the early publications following the report by Lang (101) demonstrated that many biennial plants were not very responsive to gibberellin (37). The photoperiod or the low-temperature requirement was satisfied but not both. Primarily, it was a matter of accelerating a process already in progress but at a slow rate. Gibberellin accelerated flowering only when the plants were maintained at temperatures just above those inductive for flower formation (165). It did not compensate for unfavorable temperatures during growth. Long photoperiods were essential for gibberellin to be operative except with a few plants that had little or no photoperiod requirement for flowering (31). The dosages needed to replace the cold requirement were in the order of several milligrams per plant.

Promotion of earlier seed production can potentially accelerate breeding

programs (165). Varieties with a tendency toward bolting may be recognized easily during growth. Also, the period of time needed to grow a generation of a biennial plant can be reduced by following exposure to low temperature with long photoperiods and biweekly applications of gibberellin. Weibel (194) was able to reduce the vernalization period of winter wheat as much as 28 days by application of gibberellin. This reduction is of considerable significance to the plant breeder in terms of increasing the number of generations that can be grown in one year.

Seed treatment of Kharkov winter wheat prior to planting in the fall stimulated shoot growth before freezing and reduced vigor of subsequent spring growth and forage yields (47).

Caso, Highkin & Koller (32) demonstrated that Petkus winter rye, a cold-requiring plant, could be induced to form normal flowers in the absence of vernalization by the action of gibberellic acid. The application had to be made at a proper time in the development of the plant (ten-leaf stage). Applications made at the five-leaf stage were inhibitory. The treatment was effective under long-day and short-day conditions. Purvis (152) noted similar abnormalities in flowering response when early leaves were injected with gibberellin but the flowering response following injection through the ninth leaf alone was normal and free from abnormalities. Nevertheless Purvis (152) concluded that gibberellin had no direct effect on flower induction in winter rye and therefore could not replace low temperature in this respect. The accelerating effect on flowering was explained as appearing to operate at some post-inductive stage.

Photoperiod.—Gibberellin treatments of plants maintained on non-inductive conditions induced vegetative developments similar to those following exposure to longer days. Most long-day plants kept in a non-inductive environment produce rosette plants without an elongate stem. Applications of gibberellin to all facultative long-day plants induced them to flower earlier than untreated plants (202). Upon treatment with gibberellin, certain obligate long-day plants such as spinach, endive, and pyrethrum flowered on short photoperiods but plants of one *Chrysanthemum* variety (feverfew) did not do so. When these plants were induced to flower by treatment with long days, stem elongation and flower initiation occurred almost simultaneously. In contrast, when flowering occurred on plants treated with gibberellin, it came only after a period of stem elongation (37). Also, the flowers produced on the gibberellin-treated plants often bore aborted seeds (77). Treatment of caulescent long-day plants (i.e., long-day plants with elongate stems on short days) did not alter the requirement of long days for flowering (114). The primary effect of gibberellin was on stem elongation. After a considerable duration of treatment, flower formation was brought about secondarily.

Nagao & Mitsui (129) demonstrated that the aerial tubers and flowers of *Begonia evansiana* Andr. were formed under short days while seed germi-

nation was in response to long days (128). The photoperiod response of *Begonia evansiana* Andr. (128) and *Kalanchoe blossfeldiana* (28, 29) was markedly modified by applications of gibberellin. Gibberellin increased the germination of begonia seed under continuous light but also reduced the critical daylength, even 1-min. photoperiods being inductive. Seed did not germinate in darkness even in the presence of gibberellin and the duration of a single light exposure required for germination was not shortened by gibberellin treatment. Gibberellin promoted germination only when 2 to 3 photoperiod cycles were applied to the seed.

The timing of applications of gibberellin to obtain a given plant response in long-day and short-day plants was extremely important. For China aster, applications of gibberellin had no effect on inflorescence initiation under long days, but markedly reduced the number of leaves laid down before inflorescence was initiated on plants growing under short days (55). The growth stage at time of treatment of the aster plant was more important than the number or frequency of gibberellin applications. Caulescent short-day plants, such as Maryland Mammoth tobacco (115) and chrysanthemum (36), did not flower when treated with gibberellin under photoperiods unsuitable for flowering.

The most pronounced increases in height occurred when gibberellin was applied to plants on short days (173, 204). Gibberellin treatment of plants that had recently been transferred to inductive short photoperiods led to extensive vegetative development and inhibition of flowering of Kalanchoe (28, 29) and strawberry (178). It also reduced the number of flowers in chrysanthemum (34) and *Zygocactus truncatus* (Haw.) Schumann (62). Applications of gibberellin to plants with the partially developed flowers accelerated development of the individual flowers (36, 62). Gibberellin promoted extension of the lateral peduncles of chrysanthemum from applications made immediately following flower initiation. Long days promoted a similar extension of the peduncles but it was necessary to give long photoperiods during the time of floret initiation on the capitulum (149). Extension induced either by favorable photoperiod or by gibberellin appeared similar at flowering, but the modes of action were related to quite different stages of development. The action of long photoperiod was achieved by a mechanism different from the one released by gibberellin treatment. The two mechanisms led to a point at which presumably there was a common metabolic path, eventually resulting in stem extension. Reduction or inhibition of flowering of short-day plants by applications of gibberellin was simply a matter of increasing the apical dominance of the plant, and channeling growth into stems with relatively few flowers (17).

Auxins.—Applications of various growth substances in combination with gibberellin were sometimes more than additive in their action. When indoleacetic acid was added to cuttings of sycamore, poplar, and ash, a narrow zone of new xylem with lignified vessels was formed. Gibberellin

alone induced the formation of many small unlignified cells with no new vessels. When the two substances were combined, a wide zone of new wood consisting of fully lignified vessels with the intervening fibrous tissue was formed (187). Similarly, gibberellin increased cell division in the xylem near the ray cells of apricot spur shoots (13).

Gibberellin suppressed rooting of cuttings from many plants such as pea and bean (18) and citrus (39). Small dosages applied to the base of the cutting reduced rooting without affecting stem extension. When such dosages were applied to the tip, the stems extended but rooting was not affected. In combination with indoleacetic acid, gibberellin promoted stem extension and increased the number of roots. Inhibition of rooting by gibberellin was thus entirely independent of cutting extension; rather it depended upon the place of treatment and the availability of the naturally occurring auxins. In contrast, repeated applications of gibberellin to mature stem cuttings of *Salix gmelinii* Pallas and *S. purpurea* L. had only a slight effect on the number of roots. Root primordia were already present on the cuttings. Gibberellin therefore inhibited the change of adult stems to a meristematic condition rather than inhibiting the development of roots (69).

Although an increased set of fruit as a result of application of gibberellin has been observed in tomato (205) the fruits were never as large as those induced by *p*-chlorophenoxyacetic acid and 2,4-dichlorophenoxyacetic acid (210). Application of gibberellin decreased the tendency of the fruits to develop hollow locules. Without pollination the fruits produced from application of gibberellin were seedless, smaller in size, and higher in specific gravity than normal seeded fruits. Indoleacetic acid was only moderately active in promoting growth of the fruit, but a marked synergistic action, which was more than additive, was observed when it was combined with gibberellin (116).

On agar containing 10 per cent sucrose, some germination of tulip pollen occurred at concentrations of gibberellin between 100 and 500 μg per ml. with a maximum around 300 (100). Indoleacetic acid alone did not stimulate any germination. At a sub-optimal concentration of gibberellin, 1 to 100 μg per ml. of indoleacetic acid augmented the effect. At the optimum and higher concentrations of gibberellin, all concentrations of indoleacetic acid inhibited germination. The pollen of both responsive and non-responsive varieties of grapes contained substances that were gibberellin-like in activity (44), and yet only berry development of seedless varieties was affected by applications of gibberellin.

Plants of *Impatiens balsamina* L. yielded 33 per cent single flowers. Repeated applications of gibberellin throughout growth promoted the penetrance of doubling (195). Adding indoleacetic acid at concentrations of 0.1 mg. per liter accelerated flowering by three weeks. Dosages lower than 0.1 mg. per liter of indoleacetic acid increased the stem elongating effect of gibberellin while higher dosages inhibited it.

In amounts greater than 1 μg. per plant, 2,4-D inhibited shoot elongation induced by gibberellin. Inhibition was proportional to the dosage of 2,4-D. When 1 μg. or less was used, the two compounds acted synergistically and promoted shoot elongation of cotton seedlings and petiole elongation of bean leaves (41). Gibberellin reduced slightly the weight of nutgrass (*Cyperus rotundus* L.). When applied two times, 7 days apart, with 10 pounds per acre of 2,2-dichloropropionic acid on the third day, the combination caused more visual toxicity than 2,2-dichloropropionic acid at 20 or 2 to 10 lb. per acre. The weight reduction was more than additive, suggesting synergism (79). Toxicity as measured by dry weights of foliage was increased by applications of 2,4-D and ethyl-di-*n*-propylthiocarbamate (EPTC) in conjunction with gibberellin. For maximum effect it was necessary to apply the gibberellin before applying the herbicides. Apparently the gibberellin accelerated the rate of cell division and transport of the applied herbicides rather than increasing their absorption.

Purine derivatives.—Gibberellin and 6-(substituted)-purines had different temperature ranges of activity for promoting germination of Grand Rapids lettuce seed (72). The purine kinetin was effective at 22 and 35°C., at which temperatures gibberellin was ineffective; gibberellin was effective at 7 and 17°, at which temperatures kinetin was ineffective. At 35°C., gibberellin promoted germination only in the presence of kinetin. This effect was observed even in the presence of kinetin at optimal concentration for promoting germination by itself at this temperature. At 17°, gibberellin promoted germination in the absence of kinetin but not in its presence. A combination of gibberellin and 6-(substituted)-purines acted synergistically in stimulating the rate of germination of cultivars of carpet grass, white Dutch clover, and lettuce (162, 163).

Growth-retarding substances.—Many types of compounds, synthetic and naturally occurring, have been shown to antagonize or to negate growth promotion induced by gibberellin. A quaternary ammonium carbamate known as Amo-1618 (2-isopropyl-4-dimethylamino-5-methylphenyl-1-piperidinecarboxylate methyl chloride) acted like red radiant energy in that it retarded growth of internodes of light-grown plants. It equally retarded dark-grown plants (56). Gibberellin nullified the inhibitory effect of Amo-1618 on Red Kidney beans (56) and chrysanthemum (34). Amo-1618 inhibited and gibberellin promoted cell division in the sub-apical meristems of chrysanthemums (156). These compounds were mutually antagonistic when applied as foliar sprays to the apical meristem in the proportion of 1 part gibberellin to 10 parts Amo-1618 on a molar basis (33). Reciprocity held over a wide range of concentration. Applied simultaneously to rooted cuttings, equal concentrations were necessary to antagonize the growth control (33, 156).

Substituted choline compounds, the most active of which was (2-chloroethyl) trimethylammonium chloride, antagonized the stimulation of lettuce

seed germination, the extension of the second leaf sheath of mutant dwarf-1 maize, and the stem elongation of Grand Rapids lettuce and Biloxi soybeans that were induced by applications of gibberellin (206).

Coumarin, a non-specific growth substance occurring naturally in many plant tissues, imposed a dormancy on the germination of Grand Rapids lettuce seed. Gibberellin reversed the inhibition of germination but the concentration was higher than that required for germination of untreated seeds (121).

These substances should not be called anti-gibberellins since they did not always compete directly with gibberellin. Applied alone, (2-chloroethyl) trimethylammonium chloride had no effect on the growth of tomato ovaries. In combination with indoleacetic acid and gibberellin, a growth rate more than could be attributed to indoleacetic acid-gibberellin interaction was induced. The relative inactivity of (2-chloroethyl) trimethylammonium chloride when applied as a foliar spray and the high dosages necessary to reduce materially the rate of stem extension, contrast with the low dosages required for a gibberellin response. Growth-retarding substances, particularly when assayed on germinating seeds held in the dark, retarded growth and changed the rate of emergence of the radicles. Eventually, however, growth was apparent. In contrast, gibberellin promoted lettuce seed germination immediately, and rate of emergence or length of plant parts was of minor consideration. The chemical structures of gibberellin and the growth retardants were so different that a simple system of direct competition would be difficult to visualize.

General Comments

The dramatic growth effects induced by gibberellin aroused popular interest in these materials and they have been widely applied in hopes of increasing crop yields. As more evidence accumulates it becomes apparent that gibberellins occur naturally in plants and that the beneficial activity of those applied is mediated through interaction with other regulators, auxins, purine derivatives, mineral elements, and products of carbohydrate metabolism. As shown in this review, directed applications of gibberellin to specific plants at specific times have induced some beneficial responses (Table I). On the other hand, some responses have been slight in magnitude, undesirable in nature, and demanding in timing and dosage. These responses cannot be interpreted in terms of gibberellin alone.

Broadly speaking we may assume that growth of plants is the net result of the influence of growth-promoting materials versus growth-inhibiting materials. It is observed that gibberellin often induces maximum responses when growth conditions are adversely influenced by temperature, nutrition, or other environmental factor. Possibly under these conditions synthesis of both native gibberellins and inhibitors is retarded, permitting the greater response from exogenous gibberellin. For the most part, applied gibberellin

does not establish new growth patterns but accelerates those in progress. Future applications of the gibberellins would seem to depend upon further information concerning synergists and methods of application, as well as information concerning new gibberellins and their derivatives.

TABLE I

DOSAGES AND FREQUENCY OF APPLICATION FOR PHYSIOLOGICAL RESPONSES TO GIBBERELLIN

Response	Treatment	Dosage	Frequency	Reference
		(p.p.m.)		
Germination				
Malting of barley	Imbibing solution	1–2	Once	157
Lettuce seed	At planting	35	Once	93
Lepidium seed	At planting	750	Once	181
Elongation				
Leaf sheath of single gene mutant maize	Any time during growth	0.1	—	145
Bean	Any time during growth	1	—	200
Chrysanthemum	During flower initiation	100	—	36
Deciduous trees	Prior to growth	10,000 (in lanolin)	—	113
Flower acceleration				
Cold-requiring biennial—cabbage	1st treatment at stem diameter of 1 cm. and 7 to 9 leaves	1000	8 times weekly	202
Facultative long-day plant—mustard	Plants with 8 to 10 leaves	100	3 times	202
Obligate long-day plant—feverfew	Stem elongation, no response flowering	No response	—	202
Obligate short-day plant—chrysanthemum	Florets beginning to open and showing flower color	1000	5 times daily	36
Organ maturation				
Parthenocarpic fruit-set on tomato	At flowering	6	Once	205
Flower size in geranium	Florets beginning to open and showing flower color	10	Once	109
Induction of staminate flowers—cucumber	Applied at first true leaf stage	1500	4 times weekly	143
Berry enlargement—Thompson Seedless grape	When 50 per cent of calyptras had fallen	20	Once	188
Breaking of dormancy—white potato	Freshly harvested	1	Once	110

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KINETIN AND RELATED COMPOUNDS IN PLANT GROWTH[1,2]

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Purines in some form are probably implicated in all phases of plant growth. These compounds are structural parts of several of the energy handling systems, unit transfer systems, and the genetic communication machinery. Thus, they are involved in practically all synthetic steps which occur as a cell or plant grows; and the same is likely to be true for any living thing. Because the purines have so many important roles, they have been studied from numerous viewpoints and much knowledge concerning them has accumulated. To the plant scientist interested in studying the chemical basis of growth, this availability of knowledge offers a wonderful opportunity; normal growth patterns can be correlated with biochemical information concerning purines, or the patterns can be altered through controlled application of purines and related compounds. Such alteration of growth patterns recently has been achieved to a rather great degree with the substituted purine known as kinetin. This review by no means seriously considers or even lists all the publications that deal with kinetin or related compounds in some manner. Rather, an attempt is made to bring together enough information to indicate the present lines of research dealing with such compounds. The subject has been reviewed earlier by Strong (1).

Discovery of Kinetin

The discovery of kinetin was an outgrowth of the tissue culture investigations in Professor Folke Skoog's Wisconsin laboratory. In much of this work, segments of tobacco (*Nicotiana tabacum*, var. Wisconsin #38) stems were aseptically removed from the plants and cultured on synthetic media. All tissues external to the cambium were removed and then tangential slabs were sliced from the remaining cylinders. Such slabs, when placed on a basal medium (solidified with agar) containing mineral salts, sucrose, vitamins, and glycine, formed lumps of callus tissue by cell division at their morphologically basal ends. This callus tissue initially grew quickly but growth soon slowed and stopped. The addition of IAA augmented the early growth but did not bring about continued production of tissue. The lumps of callus, when excised from the stem slabs and placed on a fresh supply of medium, neither continued nor resumed growth. Apparently some stimulus originally present in the stem segments became exhausted after a limited amount of growth. Supporting this conclusion was the fact that adding coconut milk or yeast extract in combination with IAA did promote new and continued cell division.

[1] The survey of literature pertaining to this review was concluded in June 1960.
[2] The following abbreviation will be used: IAA (indoleacetic acid).

A program was initiated to determine the nature of the active principle. Since a very ample supply of yeast extract existed, fractionations were started on this material. Each fraction was tested for activity simply by adding it to the basal medium along with 2 mg./l. IAA, sterilizing the mixture, and solidifying it with 1 per cent agar. Small pieces of tobacco slab callus that had almost stopped growing were then planted on the medium. In four or five days, the presence of the active material could be detected by its promotion of a relatively vigorous appearance of the tissue. After two or three weeks, fresh and dry weight measurements served the same purpose. The active principle could be extracted into 95 per cent ethanol and precipitated by silver salts under acidic conditions, and a small fraction of the material precipitated by silver was extractable into diethyl ether. Paper chromatography of the ether extract, in turn, yielded a spot that showed an absorption peak in ethyl alcohol at 268 mμ and its presence or absence was always correlated with activity.

These observations led to the conclusion that only one compound, rather than a mixture, was needed for activity. Furthermore, the silver precipitability and the ultraviolet absorption band formed the basis of the suggestion that the active compound might be a purine. However, the common purines had been tested and found to cause no promotion of cell division in the tobacco tissue. Since the possibility remained that a hitherto undetected purine was responsible, DNA and RNA, obviously rich sources of purines, were tried in the tissue test system. The DNA was quite active even at a low concentration but the RNA was without effect. Extracts from the DNA gave active material that could be precipitated by silver, extracted by ether, and had an absorption peak in ethanol at 268 mμ. Fractionation procedures for complete purification were achieved with the small sample of DNA and then a large batch of the nucleic acid was obtained from a commercial source for the final isolation. This large batch upon arrival had no activity, although the same company, using the same methods, had prepared both batches.

However, a few months later, large quantities of the active principle were found to be formed by autoclaving aqueous slurries of DNA under acidic conditions, and the compound was isolated in crystalline form [Miller *et al.* (2)]. Active material also was formed in inactive DNA simply by placing the acid on the shelf at room temperature for several months. In fact, the difference between the original and the new batch could be explained by this observation. That it was not originally present was indicated by the findings that acidic or enzymic hydrolysis failed to yield active material from the new nucleic acid. Obviously, the compound was formed as the nucleic acid became degraded. The findings of Hall & de Ropp (3) indicate the compound is formed from deoxyadenosine that has been freed from the DNA. Because of its ability to bring about cytokinesis in cells that otherwise might become multinuclear [Naylor *et al.* (4)], the active compound was named kinetin. Shortly afterwards, the structure of kinetin

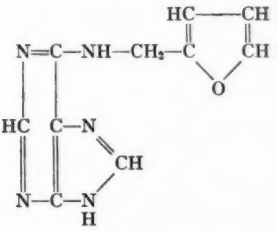

FIG. 1.

was shown to be 6-furfurylaminopurine (see Fig. 1), and it was prepared from pure chemicals [Miller *et al.* (5, 6)].

BIOLOGICAL EFFECTS

Cell division and enlargement.—As already indicated, the first effect observed after treatment with kinetin was an acceleration of cell division in the tobacco callus tissues, provided that IAA was also present. Such an effect was perhaps even more striking in tobacco pith cultures [Miller *et al.* (6), Skoog & Miller (7)]. The parenchymatous cells of this tissue exhibited very little, if any, production of new cells on the control media with or without kinetin, but did show some mitoses when treated with IAA. A combination of kinetin with IAA, however, evoked a high rate of cell division. This role of kinetin, along with IAA, in stimulation of cell division in the tobacco pith has been carefully analyzed and apparently DNA synthesis and mitosis require both compounds; a requirement for IAA in cytokinesis was not established but is still possible [Das *et al.* (8), Patau *et al.* (9)]. Slight effects obtained with either substance alone probably were dependent upon small endogenous amounts of the other.

Stimulation of cell division in essentially unorganized tissues also has been observed in carrot root tissues [Shantz *et al.* (10)], soybean cotyledon callus [Miller (11)], pea root callus [Torrey (12)], and cocklebur stem callus [Fox & Miller (13)]. Haber & Luippold (14) have observed a specific stimulation of mitosis in radicles of ungerminated lettuce seeds. In addition, several different growth effects to be mentioned later in this review can be explained by assuming a stimulation of cell production. It is obvious that kinetin can very dramatically increase cell division under certain conditions. There have been several reports, however, concerning failure of the purine to cause or increase division. For example, Jerusalem artichoke parenchyma tissue did not respond under the conditions used by two different groups of investigators [Shantz *et al.* (10), Adamson (15)].

Shortly after its discovery as a cell division factor, the 6-furfurylaminopurine was found to influence cell enlargement as well. Disks cut from etiolated leaves of *Phaseolus vulgaris* responded to kinetin by expanding [Scott & Liverman (16), Miller (17)], and this expansion was due to cell

enlargement [Miller (17), Powell & Griffith (18)]. The same was true of disks excised from light-grown leaves of *Raphanus sativus* [Kuraishi & Okumura (19)]. Adamson (15) reported an enhancement of cell enlargement by kinetin in Jerusalem artichoke storage parenchyma cultured on agar containing IAA. As mentioned above, this tissue did not respond to kinetin in terms of increased cell division. The kinetin effect was especially noticeable when the artichoke tissue did not respond very markedly to IAA or when the IAA was supplied at sub-optimal levels. Under certain conditions, an increase in cell elongation in tobacco pith sections was brought about by low concentrations of kinetin in a matter of a few hours [Glasziou (20)]. Enlargements of the cortical cells of tobacco roots occurred when the roots were exposed to kinetin [Arora *et al.* (21)]. The strong inhibition of pea stem segment elongation very likely was due to a retardation of cell lengthening [Miller (17), Brian & Hemming (22)], as was partially the inhibition of *Allium sativum* root elongation [Deysson (23)]. From these investigations and others, one may conclude that kinetin clearly can influence cell enlargement and thus is not to be regarded solely as a cell-division substance.

Shoot initiation.—The tobacco callus used in the original kinetin work could be kept in an essentially undifferentiated state through an indefinite number of sub-cultures, provided that the proper balance of auxin and kinetin, along with certain major nutrients such as phosphate and amino acids, was maintained. If the ratio of kinetin to auxin was increased, however, either by increasing the concentration of kinetin or lowering that of the auxin, leafy shoots were initiated. Similar results were observed with tobacco stem segments, although here the budding was much greater if adenine was also present in the medium (7). Danckwardt-Lillieström (24) showed that kinetin increased the regeneration of shoots from clones of *Isatis tinctoria* roots maintained in a sterile culture medium. He suggested that, since first passage roots formed shoots on the control medium but later passage roots did not, the roots while still attached to the shoot probably contained kinetin as a natural hormone. This effect was much more evident with roots grown in the light than with those grown in the dark. Torrey (25) observed that 6-furfurylaminopurine strikingly increased the number of buds formed at the root-end of *Convolvulus arvensis* root segments. In this case also light increased the responsiveness of the plant material to kinetin.

A large increase (110 per cent) of budding in *Saintpaulia ionantha* was observed by Plummer & Leopold (26) after petioles of the leaf cuttings were immersed for 24 hr. in a 25 mg./l. kinetin solution. Under control conditions, disks cut from leaves of Begonia formed roots and then shoots at the basal ends of main veins. When treated with kinetin, however, root growth was suppressed and shoots appeared sooner, in larger quantities, and all around the edges of the disks; that is, kinetin not only promoted shoot initiation but also eliminated the usual polarity of such initiation [Schrandolf & Reinert (27)].

The stimulation of bud formation is not limited to the angiosperms or

even to spermatophytic forms. Gorton *et al.* (28, 29) quite early found that bud formation on protonemata of the moss *Tortella caespitosa* was increased about eightfold by the optimal concentration of 5 mg./l. kinetin. Budding in protonemata of *Pohlia nutans* was greatly promoted by treatment with kinetin at 0.1 to 10.0 mg./l. [Mitra & Allsopp (30)]. An indication that kinetin may promote the formation of *Ulva lactuca* germlings and of normal flat blades has been obtained [Provasoli (31)].

To be kept in mind is that all of the plants reported to respond to kinetin in terms of increased budding show some formation of shoot structures even in the absence of kinetin. Thus, kinetin is known to cause bud formation only in those plant materials with an inherent tendency for such formation; the compound must not be regarded as a specific "bud-former." This area of research will become much more enlightening and fascinating as we learn how to cause budding in plant materials not so disposed.

Bud elongation.—Wickson & Thimann (32) drew attention to the fact that under the influence of kinetin many buds form on a single tobacco tissue fragment without seeming to inhibit each other's growth. These authors therefore felt that the compound might be influencing not only shoot formation but also shoot elongation and development. Their experiments with stem sections cut from *Pisum sativum* var. Alaska plants demonstrated that kinetin overcame the dominance of the apical bud over the growth of the lateral ones and negated the inhibitory effect of IAA applied to the stem segments. Wickson & Thimann suggested that normal apical dominance depends on an interreaction between auxin and a kinetin-like substance in plants. Lona & Bocchi (33) found that kinetin, when applied daily to the tops of plants, caused an inception or intensification of shoot branching in *Perilla ocymoides* var. *nankinensis*, *Centaurea calcitrapa*, *Crepis leontodontoides*, *Centaurea cyanus*, *Aster chinensis*, *Aethusa cynapium*, *Triticum vulgare*, and *Secale cereale*. The indicated similarity of physiological control of morphogenesis in mosses and angiosperms is strengthened by the findings of Von Maltzahn (34) that lateral bud activation in *Splachnum ampullaceum* is controlled by the apical region of the gametophore, that IAA effectively substituted for this region and that kinetin overcame the inhibition due to the IAA. Inhibition of basal regeneration either by the IAA or by the apical region was not overcome by kinetin, however.

Root growth.—There exists only rather meager evidence that kinetin under certain circumstances may promote initiation of roots. In the presence of casein hydrolysate and IAA, kinetin at only 0.02 mg./l. apparently increased the amount of rooting from tobacco stem callus and also accelerated the growth of the roots formed (7). Higher concentrations of the purine inhibited both the formation and growth of roots, undifferentiated callus being formed at medium concentrations, and budding being promoted at the highest levels. Using intact seedlings of a strain of *Lupinus hartwegii*, Fries (35) demonstrated an enhancement of lateral root growth when the roots were in a medium containing about 3×10^{-7} *M* kinetin. Arora *et al.* (21) studied the anatomy of pseudonodules which appeared on tobacco roots

under the influence of kinetin (about 0.2 mg./l.) and concluded that although the structures arose from cortical tissues, they bore no anatomical resemblance to the typical nodules caused by *Rhizobium*. DeRopp (36) observed only inhibition of rooting by kinetin (0.01 to 1.0 mg./l.) applied to sunflower hypocotyl sections. An unusual stimulation of root formation on sporeling leaves of the water fern *Marsilea drummondii* was obtained at a concentration of 0.01 mg./l. kinetin [Allsopp & Szweykowska (37)].

The several investigations revealing an inhibitory effect of kinetin on root elongation include those with tomato root [Skinner & Shive (38)], roots of *Brassica campestris* (19), *Allium cepa* [McManus (39)], *Allium sativum* (23), and intact tomato roots [Kemp *et al.* (40)]. The investigator looking for stimulatory effects of kinetin on the formation of roots should always include low concentrations in his series of treatments so that results will not be obscured by simple inhibition of elongation.

Leaf growth.—In the discussion of cell growth the enhancement of leaf disk enlargement has already been noted; bean and radish leaf fragments respond quite strikingly to fairly high concentrations of kinetin. To these two plants whose leaf tissues respond positively to kinetin may be added *Nicotiana tabacum*, *Zea mays*, *Helianthus annuus*, *H. tuberosus*, *Solanum tuberosum*, *Pisum sativum*, *Brassica campestris*, *Spinacea oleracea*, and *Zelkowa serrata* [Kuraishi (41)]. The results remind one of the effect of adenine on radish leaf disk enlargement observed by Bonner & Haagen-Smit (42) some years ago. However, whereas adenine is effective only on disks from young leaves, kinetin increases enlargement even in disks taken from old leaves (41). Humphries (43) has reported a depression of primary leaf expansion in dwarf bean plants.

The influence of kinetin is not restricted to stimulation or depression of enlargement however. Kinetin treatment produced leaves with unusually short petioles or unusually long lobes, modified leaves which branched repeatedly, and non-laminate leaves in *Marsilea drummondii* (37). Leaf formation on apices of *Dryopteris austriaca* was slightly stimulated by a low concentration (0.1 mg./l.) of kinetin but inhibited by higher concentrations [Wardlaw & Mitra (44)]. Apparently the inhibition was due to the promotion of cell division and parenchymatous enlargement throughout the whole apical region. Kemp *et al.* (40) observed atypical leaves in tomato plants given drench treatments of kinetin.

Breaking of dormancy.—Promotion of seed germination by kinetin in several varieties of lettuce (*Lactuca sativa*) has definitely been established (e.g., 17, 45 to 49). That this stimulation is due to increased cell enlargement is indicated by the fact that germination, which has been inhibited by ionizing radiation, can be partially reversed by treatment with kinetin and under such conditions germination occurs before mitosis becomes evident in the root [Haber & Luippold (50)]. However, these authors (14) have also established that kinetin can cause mitosis in ungerminated lettuce seed radicles so the normal effect of kinetin may be due to its combination of influences on cell division and enlargement. Also to be considered in any explanation of the

kinetin effect on lettuce seed germination is the knowledge that enlargement of any part of the lettuce embryo sufficient to cause even slight splitting of the coats around the embryo will bring about germination. Seeds of tobacco (41), white clover (47), and carpet grass (47) also respond to kinetin or close derivatives. Under natural conditions, the germination of seeds of the parasitic *Striga asiatica* (witchweed) depends upon material secreted by roots of the host or certain other plant species. According to Worsham *et al.* (51) kinetin has been found to stimulate the germination of these seeds although there is no evidence that kinetin or a similar compound is the substance given off by the host plants.

The rest period of *Hydrocharis* winter buds [Kurz & Kummerow (52)] and the dormancy of *Lemna minor* [Deysson (53)] may be broken by kinetin.

Some other growth effects.—Stimulation of growth of intact plants has been demonstrated in experiments with duckweeds. Hillman has obtained promotion of frond production in *Lemna minor* in darkness but not in light (54) and Deysson (53) also has observed increased frond production in this species. Hillman (55) found that a strain of *Wolffia columbiana* required either kinetin (10^{-5} *M*) or a yeast extract-tryptone supplement to grow in the light on Hutner's medium. In a $\frac{1}{5}$ dilution of Hutner's medium, however, kinetin had no influence on growth. Hillman suggested that the kinetin overcame toxic effects of a concentrated medium. Fries (35) detected an overall increase of dry weight in intact lupine seedlings grown in darkness in response to kinetin, but very little or no response to kinetin in the light. A low concentration (about 10^{-7} *M*) was necessary since much higher levels caused distortion of growth. Adenine at about 10^{-4} *M* also was stimulatory.

Of considerable interest are the stimulation of *Avena* coleoptile growth [Shrank (56)], the inhibition of sunflower hypocotyl segment elongation (36), the promotion of growth in *Escherishia coli* [Kennell (57)], the reversal of penicillin inhibition of *Avena* segment elongation [Platt (58)], the selective activity of kinetin in promoting population changes from non-virulent (non-S) to virulent (S) cells in cultures of *Brucella abortus* and in pneumococcus cultures [Braun *et al.* (59)], the development of excised anthers of *Allium cepa* on a medium containing kinetin [Vasil (60)], the selective action shown by kinetin in promoting division of tetraploid but not of diploid nuclei of pea root tissues (12), and the increase of the mitotic index in cultures of *Polytoma uvella* [Moewus (61)].

Biochemical effects.—Although the mechanism of kinetin action is so far unknown, some rather fascinating information has been obtained about its biochemical effects. Glasziou (20) reported an increase of respiration accompanying cell elongation in tobacco pith slices within 3 to 8 hr. after the start of kinetin treatment. Guttman first suggested (62) and then obtained indications (63) that kinetin caused a quick increase in the amount of RNA found in nuclei of onion roots. Jensen & Pollock (64) reported that kinetin caused an increase of RNA and DNA in onion root cells but that protein content was unaffected. Perhaps in contrast to this, protein content in a leaf undergoes a rapid decline soon after the leaf is detached from a plant, and

this decline may be largely prevented if the leaf is treated with kinetin. At least this was true in leaves of *Xanthium pennsylvanicum* [Richmond & Lang (65)] and *Nicotiana rustica* [Mothes *et al.* (66)]. This effect of kinetin very likely is related closely to the ability of kinetin to cause at its point of application a mobilization of tagged glycine applied elsewhere on the leaf (66).

The influence of the purine on RNA may be responsible for its ability to promote protein synthesis such as that which has been shown in pea stem segments and lateral buds on such segments [Thimann & Laloraya (67)]. An attractive suggestion made by the authors just mentioned (67) is that kinetin, when applied to plant tissue, perhaps becomes fixed into a complex compound such as RNA. This would be consistent with known effects of kinetin on RNA and proteins and would also account for the high degree of localization of kinetin effects (66, 67). The acceleration of both ribonuclease and deoxyribonuclease activity by kinetin in extracts of *Phaseolus vulgaris* conceivably could be related closely to the above effects [Maciejewska-Potapczyk (68)].

Other effects of kinetin on metabolism include an increase of anthocyanin formation in cultured petals of *Impatiens balsamina* [Klein (69)] and of a red pigment in *Amaranthus retroflexus* seedlings [Bamberger & Mayer (70)]. Also, Konar (71) has noted an increased accumulation of starch when kinetin was used to promote growth of *Pinus roxburghii* pollen tubes.

Structural Requirements for Activity

Immediately after the chemical identification of kinetin, various programs were initiated to learn the structural requirements for activity and to seek even more potent compounds. Such programs have employed cell division in tobacco callus tissue [see (1)], germination in lettuce seeds (e.g., 45, 46, 47), and cell enlargement in leaf disks of radish (42), among others. We shall make no detailed listing of results here but simply mention some points of agreement. All the works agree that the furfuryl group attached to the amino group on the 6 position of the purine ring may be replaced by a rather wide variety of substituents. The furan ring, for example, may be replaced by a benzene ring to give 6-benzylaminopurine. This compound shows activity comparable to kinetin in all three tests and may be somewhat superior. Various other groups such as thenyl, tetrahydrofurfuryl, pyridyl, naphthyl, cyclohexyl, piperidyl, and chlorine-substituted benzyl can be used to make effective compounds. The replacement does not necessarily need to be a ring since 6-alkylaminopurines are quite active. For example, the *n*-hexyl-, *n*-amyl-, and isoamyl- derivatives are very effective while the *n*-butyl- and *n*-propyl- compounds are less so. Ethyl- is slightly effective and the methyl-member of this series shows little or no activity while the higher numbered members such as *n*-heptyl- and *n*-octyl- also show lower effectiveness. From the results thus far published, one may surmise that the function of the furan ring in kinetin is to endow the compound with a degree of fat solubility and that a wide range of substituents may serve the same purpose. The methyl

group between the furan ring and the amino group does not appear to be necessary since 6-phenylaminopurine is a very active compound.

The necessity of the amino group has been investigated by replacing it with a sulfur atom and it is obvious that the amino group is not absolutely essential. The compound 6-benzylmercaptopurine, for example, has been tested in the tobacco callus, radish leaf disk, and lettuce seed systems. It showed high activity in the seed germination test, a low but definite activity in the callus test and little if any activity in the leaf disk expansion system. Substitution by a mercapto for the amino group therefore modifies activity and the degree of modification depends upon the biological system. Substitution of more than one group on the amino substituent apparently lowers effectiveness.

In most investigations, the need for an intact purine ring has appeared absolute. Replacement of this ring with many others has led only to inactive compounds. Even the pyrazolo compounds, formed when the nitrogen and carbon atoms at positions 7 and 8 in the purine ring are interchanged, are inactive. However, some effectiveness in the tobacco callus test was retained when an amino substituent was added at the 2 position of kinetin or 6-benzylaminopurine (1). The one real exception which has been reported concerns 8-azakinetin in which the carbon at the 8 position was replaced by a nitrogen atom. This compound promoted cell division in carrot (10) and soybean (11) callus cultures.

Role of Kinetin-Like Compounds in Plants

Occurrence of kinetin.—Kinetin was first isolated from herring sperm DNA and later was detected in DNA prepared from calf thymus (2). Neither batch of DNA showed activity before the autoclaving treatment. A compound very similar if not identical to kinetin was partially purified from yeast extract; here, one should remember that the procedures for preparing such extracts might cause the formation of a kinetin-type compound. At the moment, there is no definite published evidence that a purine possessing growth promoting abilities comparable to kinetin exists naturally in plants or animals.

There are several reasons, however, for suspecting that such a compound may in fact occur naturally. First of all, several plant extracts bring about cell division in the same excised plant tissues that respond to kinetin. Coconut milk, coconut meat, and maize endosperm are well known sources of such extracts. Recently, the group with which the late Peter Goldacre was associated has been using young apple fruits as a source of effective extracts (72). The work of Jablonski & Skoog (73) demonstrated that some substance present in excised internal phloem can bring about cell division in blocks of tobacco pith. It may be that none of these situations involves a kinetin-type compound, but we cannot know until complete analyses have been made.

That substances very closely resembling active synthetic compounds do

occur in plant materials should be considered next. Thus, 6-methylaminopurine and 6-dimethylaminopurine are reported to be present in wheat germ [Dunn & Smith (74)], and more recently the 6-methylaminopurine has been found in soluble RNA from *Escherichia coli* [Dunn *et al.* (75)]. Both of these derivatives exhibited only a low order of effectiveness in the leaf disk system (41) and were reported to be without activity in the tobacco test (1). The important point in the present discussion, however, is that plant cells do in effect add alkyl groups to the amino group of 6-aminopurine. If, under certain circumstances, ethyl or propyl, or longer alkyl groups are added, then a kinetin type substance having kinetin type activity will be the result. It remains to be seen whether or not the cells actually do this.

Until the actual occurrence of a kinetin-type compound is demonstrated in plants, discussion of its role is perhaps meaningless. An understanding of how applied kinetin brings about its effects, however, could greatly aid in understanding plant growth in general.

Significance of structure.—Kinetin (6-furfurylaminopurine) may be regarded as a substituted adenine (6-aminopurine). Since adenine shows a definite but low order promotion of bud formation in tobacco stem segments, of expansion of radish leaf disks (41), of initiation of buds in moss protonemata (28, 29), and of growth in intact lupine seedlings (35), whereas kinetin is much more effective in all these tests, kinetin may be regarded as a highly effective adenine. It may be highly effective because of the presence of the fat soluble substituent attached to the amino group [see Strong (1)]. This fat-soluble group may permit entry into, or orientation within, a certain part of the cell in which reactions controlling growth take place.

Haber & Luippold (76) argue against this idea because the compounds in which a sulfur atom has replaced the amino group are quite active. They reason that the mercapto compounds may be regarded as derivatives of 6-mercaptopurine, and, furthermore, since this compound usually inhibits growth and is considered an antimetabolite of adenine, the sulfur analogs of kinetin should actually inhibit growth if the idea that kinetin is simply a very effective adenine is correct. This argument disregards the considerations that the idea under attack is based on observations obtained with exogenously supplied adenine and that when adenine is so supplied it might conceivably inhibit certain cellular reactions involving adenine derivatives. There certainly is no proof that the effects on growth obtained with adenine result from the adenine being used in a normal biochemical sequence. Likewise, there is no proof that kinetin is effective because of any direct accelerative involvement in a reaction necessary for growth. In fact, when one looks over the large variety of compounds active in the sense that kinetin is active, the temptation is great to think that the compound accomplishes its effects by inhibiting some reactions.

That kinetin can influence reactions involving adenine is indicated by results obtained in studies of budding on tobacco stem segments. Segments placed on a medium containing 0.5 mg./l. kinetin formed only a few buds and these were inhibited in their growth; likewise, the growth of the basal

callus was greatly retarded by the purine. Addition of adenine (54 mg./l.) not only overcame this inhibition but also allowed a stimulation of budding much greater than was obtained with adenine alone [Skoog & Miller (7)]. Thus, apparently at least two influences of kinetin existed, one promoting bud initiation and growth, and the other inhibiting growth in general. The general inhibition was overcome by adenine. It may be that the promotive influence of kinetin was not affected by adenine simply because the adenine did not get into the site of action or, if it did get to the site even in small quantities, adenine acted in the same direction as kinetin. A very interesting study might be possible seeking a correlation between such multiple effects and the structure of the kinetin-type compound; e.g., 6-phenylaminopurine alone promoted rather than inhibited the initiation of buds and growth in general on tobacco stem segments and, furthermore, the effectiveness of the compound in promoting initiation and growth was not affected by adenine (7).

The compound 1,3-diphenylurea, which Shantz & Steward (77) believe to occur naturally in coconut milk, causes some cell division in the tobacco callus system used in the isolation of kinetin; however, the effect seemed delayed, somewhat sporadic, and occurred only at rather high concentrations in comparison to kinetin (1). Because of this activity, one must wonder about the relationship of the diphenylurea to kinetin. Although the relationship has not been investigated, the idea that the diphenylurea serves as a precursor for a kinetin-type compound is tempting. Ordinarily, the cell builds up the purine ring by incorporating carbon dioxide into the 6 position. If the diphenylurea or a close derivative were to supply the carbon for this position, a purine ring with a phenylamino group already attached might result. This possibility has not been checked.

Relationship of kinetin to auxin.—That kinetin is not to be considered as just another auxin is obvious from the very first biological effect discovered. Cell division in tobacco callus tissue required both kinetin and indoleacetic acid, and neither substance supplied without the other caused continuous division and growth of the cells (1, 7). This has proved to be true in tissue cultures of other plant species. In these cultures, the two substances seem to work together but in other growth tests they seem opposed. Thus, whereas auxin promoted extension of pea stem segments (17, 22) and sunflower hypocotyl segments (36), kinetin prevented the extension. In the sunflower hypocotyls, however, the kinetin had no effect on the increase of fresh weight due to IAA and actually increased it when the IAA was missing from the medium. In many different test systems, auxin has increased the initiation of roots whereas kinetin has inhibited it except when used in very low concentrations. In general, budding has been inhibited by applied auxin and promoted by kinetin. Likewise, the onset of bud elongation has been inhibited by auxin and promoted by kinetin. In this connection, Thimann & Wickson (78) have suggested that bud growth may be due to the action of a kinetin-like substance whose synthesis is repressed by auxin, or that small amounts of auxin inhibit the synthesis of auxin itself and this is opposed by

kinetin. These same authors (79) more recently have obtained some evidence that kinetin may somewhat inhibit the uptake of IAA by pea stem segments.

Relationship of kinetin to light effects.—The expansion of dark grown bean leaf disks (16, 17, 18), the production of *Lemna minor* fronds (54), and the germination of certain varieties of lettuce seeds (17, 45, 48, 49), all are promoted by exposure to red light or by treatment with kinetin in the dark. On first consideration, the assumption that kinetin can replace the red light and therefore that the two are acting upon a common biochemical mechanism seems perfectly logical. Although this may be true if one is willing to define the biochemical mechanism in sufficiently broad terms, there is evidence that they are influencing different parts of the growth machinery. Optimal concentrations of kinetin brought about only slight germination of lettuce seeds in the dark but became much more effective when the seeds were exposed to intensities of light insufficient to increase germination in controls materially [Miller (80)]. The fact that kinetin promotes cell enlargement in bean leaf disks whereas red light promotes cell division also argues against a true substitution of red light by kinetin (18).

A likely explanation of observations in which several different agents bring about the same apparent morphological effect is that growth can be pushed from several different directions, thus influencing the same general limiting condition, or that the morphological effect when examined in fine detail can be shown to be produced by quite different actions. (Thus, for example, a cell may enlarge because of a weakening of the cell wall, an increase of cell wall material, or a purely osmotically induced water uptake.) Attempts to affect flowering in photoperiodically sensitive plants with kinetin have produced only meager results. Lona & Bocchi (33) claimed a reduction in the number of cycles needed to induce flowering in *Perilla ocymoides* when the plants were sprayed with solutions of kinetin. Isolated buds of *Perilla nankinensis* produced no flowers on a control medium under long days but did flower when supplied either kinetin or adenine [Chailakhyan & Butenko (81)]. This influence of kinetin was exerted only before the shoots had fully expanded their leaves.

Comments

The demonstrations of effects on plant growth probably will multiply in the future and add to the interest as well as the literature in this subject. This reviewer hopes that certain other aspects will receive full attention in the meanwhile, however. The work will have much more meaning if a kinetin-like substance is demonstrated in plants and methods are brought forth that will permit careful analyses of the substance in different parts of the plants and in different phases of growth. With techniques now available, the demonstration of the material—if it exists—would seem to require more labor than brilliance. Even if such a substance is never identified in plant material, the investigations seeking to elucidate the action of applied kinetin could add greatly to our understanding of the biochemical processes behind the morpho-

logical expressions of growth. Attempts to explain kinetin action in terms of its interactions with auxin, light, or other factors are fine except that they do not go far enough. Because of its relationship to the important purines and because the knowledge of these compounds is a rich and dynamic one, the chances seem excellent for the plant scientist to reveal the actual biochemical role of kinetin. Knowledge of this type could help bring plant growth studies to a new and higher level of interpretation.

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GEOTROPISM IN ROOTS AND SHOOTS[1,2]

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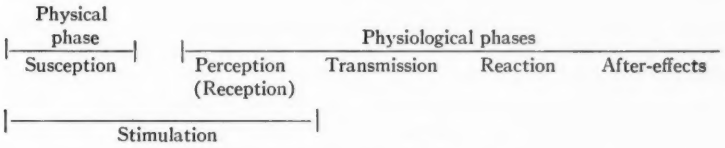
Introduction—Terminology

The last report on geotropism appeared in the *Annual Review of Plant Physiology* in 1954 (17). More recent articles have been published in *Fortschritte der Botanik* (30) and will soon appear in *Encyclopedia of Plant Physiology* XVII/2 under the names Anker, Brauner, Kaldewey, Larsen, and Rufelt.

It seems to the reviewer to be very important that a uniform terminology is used in papers dealing with geotropism. Thus the definitions of Larsen (43) will be followed in this review. According to Larsen the following phases can be distinguished in the reaction chain:

"(*a*) At least one physical phase, which may be called *susception*. (*b*) Several physiological phases.—The first physiological phase may be called *perception* or *reception*. During this phase a certain '*excitation*' is created. At a later stage, a phase of transmission of this 'excitation' to the site of the reaction can frequently be distinguished. Still later comes the *reaction* proper, for instance a geotropic bending, and the reaction may be followed by *after-effects:*



"The term *stimulation* as commonly used seems to comprise the physical phase and the first physiological phase" (43).

In several connections it is necessary to make a distinction between the geotropic reaction and a geotropic bending tendency. This is especially true in plagiotropic organs where the liminal angle is interpreted as being determined by the interaction of one positive and one negative bending tendency. A bending tendency then, often is not followed by a visible curvature or reaction but only decreases the reaction in the opposite direction. Rufelt (61, 64) has suggested the term geotropic *response* for a geotropic bending

[1] The survey of literature pertaining to this review was completed in August 1960.

[2] The following abbreviations will be used: IAA (indole-3-acetic acid); 2,4-D (2,4-dichlorophenoxyacetic acid).

[3] The author wishes to express his thanks to Dr. Paul Larsen for valuable suggestions and to Mrs. Margaret Jarvis, Ph.D., for linguistic correction of the manuscript.

tendency. A response may or may not give rise to a reaction and if a reaction can be observed, it may consist either of curvatures or straightenings of already present curvatures.

Methods

The reaction.—During the past years several new methods for studies of geotropic reactions have been described. Mostly the curvatures are photographically recorded and the angles are measured microscopically. Larsen (39, 41) has used roots of *Artemisia* grown between agar plates. This way it is possible to avoid the irregular distribution of water, which is the main risk in experiments performed in moist air. The plants are easily handled and can be rotated on a klinostat. Audus & Brownbridge (6, 7) and Rufelt (59, 60) have followed the reactions of roots immersed in aqueous solutions. The advantage in this method is that the roots can be continuously treated with growth substances dissolved in the root medium. Audus and Brownbridge have rotated the roots on a klinostat while Rufelt has not. Brumfield (21) supplied roots with auxins by immersing them in a solution, but the geotropic stimulation and the reaction took place in moist air.

Anker (1) has studied the effect of auxin supply to *Avena* coleoptiles, using a method in which the coleoptiles were severed from the endosperm, decapitated, and then slipped over thin pins of stainless steel. The pins could be moved easily from the vertical to the horizontal position and also placed at oblique angles to the plumb line. During the experiments the coleoptiles were immersed in water or auxin solution. Anker's method was also used by de Wit (73), Bottelier & Roosheroe (14), and de Boois, Bottelier & Zandee (13). The experimental time is normally only 75 min. and is made short in order to avoid the occurrence of the well known phenomenon of regeneration of a physiological tip, which takes place in decapitated coleoptiles. Short-time tests are advantageous since adaptation phenomena under these circumstances are of less importance. A disadvantage is that elongation and curvature changes are very small. In the course of 75 min. the coleoptiles grow about 1 mm. when auxin is supplied and half a millimeter without the supply. Changes in growth have often been expressed as a percentage of the control and may be about 10 per cent only. Consequently the measured difference is about 0.1 mm. According to Anker the increase in length could be estimated with an accuracy of 0.1 mm. and in such cases it seems to the reviewer that a statistical analysis should be published. Such an analysis is also highly desirable for experiments where changes in curvatures are of the magnitude of one degree or less. The angles are measured with a protractor.

A method similar to that of Anker was used by Brauner & Appel (19). Excised *Avena* coleoptiles were slipped over glass pins. The coleoptiles were not decapitated but the tips were dissected into two halves by an incision in which a mica plate was placed. Other methods used by Brauner will be described in connection with the experimental results. Rotating roots on a klinostat, Larsen (41) has found that rotation velocity affects growth as well

as the development of a geotropic curvature. Growth is decreased by rotation on a horizontal klinostat axis with the decrease most pronounced at relatively low rotation velocities. An increase was not recorded. At low velocities of rotation the stimulations in all directions are sufficient for the production of curvatures and consequently the reactions follow the motion of the klinostat. At relatively high velocities irregular curvatures are formed, i.e. curvatures not induced by the gravitational stimulus. A curvature already induced is developed. At intermediate velocities the irregular curvatures are not formed and the roots remain straight. A developing curvature is straightened. Larsen concludes that "the effect of rotation on the direction of growth of the roots depends on the magnitude of the individual stimuli, active during one half revolution,—not on their sum, which is independent of rotation velocity. The omnilateral stimulation decreases with increasing rate of rotation." Consequently, in order to allow the geotropic reaction to develop freely, i.e. without gravitational stimuli, one has to rotate the roots rather swiftly; Larsen recommends one revolution per half minute. At this velocity the centrifugal force is negligible. Thus the velocity of rotation used in an experiment is of great importance in interpreting the results. Frequently, however, the velocity is not reported. Contrasting with the results of Larsen, Winstantley (72) failed to find any effect of rotation on elongation growth.

Presentation time.—According to the definition, presentation time is the duration of a stimulation that is required just to produce a reaction. The classic method of determining the presentation time is to find the stimulation time required for the development of visible curvatures in 50 per cent of the plants used in the experiment. Normally, investigators have fixed a certain degree of curvature that must be reached before the organ is considered geotropically curved. Larsen (40, 41) has criticized this method. In experiments with *Artemisia* roots he found that small curvatures were formed even in unstimulated roots. The curvatures were up to five degrees, positive or negative. Roots stimulated for half a minute and then rotated parallel to the horizontal klinostat axis before the recording of the results formed a slight positive curvature. The aforementioned spontaneous curvatures were increased at the same time. Larsen states that if a sufficiently fine observation technique is used, 50 per cent curvatures can always be recorded. The method of recording only the curvatures that have reached a certain magnitude will not give a true presentation time but a time that is too long. As a correct method he suggests that the mean curvature produced by a certain stimulation should be determined for a series of different stimulation times and then the truly minimum reaction can be obtained by extrapolation. Larsen's method undoubtedly seems the best one for a true determination of the minimum reaction. Nevertheless, Rufelt (61) has defended the classical method. Both methods provide a linear connection between the intensity of the stimulation and the magnitude of the curvature; thus, in experiments where measurements of changes in the presentation time are intended, both methods can be used. In experiments where only the truly minimum reaction

is to be determined, the method of Larsen is the best one. On the other hand, changes in the presentation time induced by different conditions will give more information about the perception processes than its absolute value and thus the classical method of determination, which is easier and faster to perform, may be preferred in many cases. However, in comparing presentation times obtained in different experiments one has to consider the method used. This is hardly a new complication because values obtained for presentation times as well as for reaction times have always depended on the method of determination.

Perception.—It is well known that we have at present no method for the quantitative determination of the stages in the reaction chain that precede the reaction. It has been suggested that the geoelectric effect produces the unequilateral distribution of auxin. The possibility of finding a method to determine such an early stage has probably inspired several recent investigations. The methods of measurement used earlier by Brauner have been criticized. The criticism has been extensively discussed by Brauner (18) and will not be reviewed here as it has more physical than physiological interest. In a recent preliminary report, Hertz (33) described a new electrostatic method of measurement whose main advantage is that no electrodes touch the plant organ. The results obtained with this method are readily reproduced; however, it can be argued that only the sum of the potential differences is measured. Lundegårdh (44) earlier found that the potential difference in the extreme tip of a root has a sign opposite to that in the elongation zone and, further, that opposite signs are obtained with applied and inserted electrodes. The latter result is interpreted by Lundegårdh as showing that the potentials on the surface and within a root are not identical. So subtle an analysis cannot be performed with the method of Hertz.

The Course of Geotropic Reactions

Roots.—Larsen (41) has investigated the course of the geotropic reaction of horizontally placed roots of *Artemisia absinthium.* During the first 70 to 100 min. they curve at an approximately constant rate. Later the rate of curvature is reduced. The reduction is interpreted as the result of a decrease in the geotropical stimulation that will become gradually weaker with the decrease in the stimulation angle. The connection between the intensity of stimulation and the magnitude of the reaction is expressed in mathematical terms and the curve obtained is compared to the time curve (the geotropical curvature at different points in time plotted against time). The theoretical curve is found to fit closely with that experimentally obtained during the first 200 min. A depression in the experimentally found curvature is obtained later. Larsen suggests that this depression is "caused by a decrease in geosensitivity or reactivity, or it may be the result of an 'antireaction'." Roots rotated on a horizontal klinostat axis after the stimulation, the velocity being one revolution in 0.5 min., give curvatures of the same type as those continuously stimulated. Working with pea roots Audus & Brownbridge (6, 7)

have recorded the curvatures obtained after 40 min. of stimulation in a horizontal position. The roots were rotated parallel to the horizontal klinostat axis before the stimulation commenced and during the development of the curvature. The positive curvature which developed during the first 2 hr. subsequently diminished and after about 5 hr. the roots were straight again. The growth rates of the concave and the convex sides were determined by measuring their length at intervals of one hr. At the beginning of the reaction elongation was inhibited on the lower side. Simultaneously a slight stimulation could be recorded on the upper side. The stimulation was soon changed to an inhibition on the upper side too. Recovery to the initial growth rate was faster on the lower side. The straightening of the curvature was interpreted by Audus and Brownbridge as autotropic but Larsen (43) has doubted that this interpretation is correct and has claimed that it can also be explained as a result of omnilateral stimulation on the klinostat.

Rufelt (60) has recorded the course of the geotropic reaction of non-rotated wheat roots. The curvature is quantitatively different under different environmental conditions but can be described as follows. A positive curvature starts at the extreme tip of the root and is later more or less straightened at the tip, but migrates backwards within the elongation zone. The first stages of the positive reaction and the straightening in the negative direction are probably not true growth reactions but are more or less reversible. The later stages of the positive reaction are truly irreversible. A negative irreversible curvature is formed in the apical part of the extension zone after a few hours (the time of appearance varying with temperature). This negative reaction was found to occur when the cells, localized within the meristem at the start of the stimulation, had reached the elongation zone. The negative reaction was found not to extend basipetally in the extension zone and therefore the roots were finally S-shaped. The irreversible reactions were found to be caused by growth inhibitions on the concave sides that were more pronounced than any growth stimulations that might have occurred on the convex sides. The mutual strength of the positive and the negative bending tendencies (denoted positive and negative responses by Rufelt) determined the course of the geotropic reaction and could be varied by changing the environmental conditions. The roots curved more positively at higher pH values (7 to 7.5) than at low (5 to 6) (61), at low temperature (10°C.), and with a high oxygen supply to the root medium (62). In all cases it was found probable that the negative response was affected more than the positive one.

Wheat roots were found to be plagiotropic organs, and when placed in the vertical position they changed their growth direction and deviated from the plumb line. The angle of deviation could be changed in different ways and was found to be more acute at low temperatures and at high pH values, i.e., the roots grew more steeply downwards under these conditions. A sudden increase in the pH value of the root medium was found to produce a downward curvature in the roots. This curvature was of the same type as the irreversible negative reaction since it did not extend basipetally in the extension zone.

The angle of deviation could also be changed by certain growth regulating substances. Under all conditions investigated, there was a parallelism between the magnitude of the deviation angle and the strength of the negative reactions.

Ching, Hamilton & Bandurski (24) have followed the course of the geotropic reaction of roots and shoots of corn and pea and of *Avena* coleoptiles. The time intervals between the records were too great to permit the drawing of time curves except in the case of corn roots. Here the positive curvature is depressed 4 to 5 hr. after the beginning of the reaction. The depression of the curve is overlooked by the authors but it is quite visible and is greater than the standard deviation of the measurements from which each point of the curve is derived. Brumfield (21) found a similar depression of the positive curvature in *Phleum* roots.

The existence of bendings of roots in the negative direction is very interesting in the light of the concept accepted by several students of geotropism in the twenties. First, plagiogeotropic organs were considered to grow in their liminal direction because of one positive and one negative geotropism acting in the opposite directions. Second, orthogeotropism was assumed to be nothing but a special case of plagiogeotropism. (The problems are more fully discussed by Rufelt in *Encyclopedia of Plant Physiology*, XVII/2.)

Bennet-Clark, Younis & Esnault (9) have recorded the geotropic reaction produced when a root growing in moist air is placed vertically with the tip pointing upwards. They were able to confirm the earlier results obtained by Nêmec (49) and Zimmermann (75) that a root placed in such a position is unable to complete a curvature all the way back to the normal vertical direction with the tip downwards. Bennet-Clark *et al.* found the rate of curvature to be rapid during the earliest stages. After 3 to 7 hr. the bending became very slow and the roots grew at an oblique angle to the plumb line. If the roots which had reached this stage were turned until the tip again pointed vertically upwards a new positive reaction began and developed in the same way as the first one. Growing in soil or in sand the roots did not finish their positive reaction before they had reached the normal vertical line. This fact was explained as the adding of a haptotropic (or touch) stimulation to the geotropic one. A considerable range of different patterns was found to exist in different species. (Roots of mustard, pea, and bean did not complete their curvature into the vertical position in moist air but roots of *Linum usitatissimum*, *Gilia capitata*, and *Artemisia sp.* did.) Bennet-Clark *et al.* have suggested an explanation not in accord with that accepted by the earlier authors. The former suggest that the change of position releases a dose of hormone, which produces the changes of growth causing the geotropic reaction. The decrease in rate of curvature is a consequence of metabolic break-down of the hormone. Earlier authors suggested that the roots became plagiotropic. They suggested that a negative geotropism was induced in the root and this was counteracting the positive one. In fact, this latter opinion seems to be supported by the results of Bennet-Clark *et al.* In the transition between the rapid phase and the slow phase of the geotropic bending, the individual roots

are reported to "reveal to and fro bending movements." These movements are assumed to be autotropic, but it is also claimed that we have no physiological or chemical picture of autotropism. It seems to the reviewer that the movements could be explained as the result of a counteraction between a positive and a negative geotropism.

Shoots.—It was also suggested that the hypothesis that orthogeotropism is a special case of plagiotropism is valid also for shoots. Support can be found in recent experiments. Palmer (51) found the prostrate growth of runners and shoots of some tropical plants, *Alternanthera ficoidea* Moq., *Commelina cayennensis* Rich., *Cynodon dactylon* (L.) Pers., *Echinochloa colonum* (L.) Link, *Eleusine indica* (L.) Gaertn., *Mimosa sensitiva* L., *Panicum purpurascens* Raddi, and *Portulacca oleracea* L., to be caused by a photo-induced plagiogeotropism. All species became erect when they were darkened. Palmer suggests that illumination produces an increased positive geotropism that counteracts the normally dominating negative one. He is further of the opinion that the positive geotropism is affected more easily than the negative one. We would then have a behaviour quite similar to that of wheat roots; the weakest geotropism is the most labile one.

Time curves of the geotropic reactions of *Avena* coleoptiles and *Helianthus* hypocotyls have been published by Bara (8). No sign of a counter-reaction is found in *Avena* coleoptiles, but some irregularities in the curve of *Helianthus* could possibly be taken as an indication of the existence of a positive geotropism. Of course other explanations can easily be found, but the irregularities are interesting in connection with a result obtained by Brauner & Hager (20). They found that *Helianthus* hypocotyls, stimulated in the horizontal position and thereafter placed vertically for 10 min., curved negatively during the following 90 min. on a klinostat. After that the curvature was straightened and a curvature in the positive direction was formed. In decapitated hypocotyls the positive curvature failed to appear even when IAA was supplied to the hypocotyl via the cut surface. De Wit (73) reports similar results using *Avena* coleoptiles. The experiments confirm earlier observations by Dolk (26). According to him, coleoptiles decapitated immediately after a geotropic stimulation begin to curve in the same way as intact controls; however, no straightening appears. When a physiological tip is regenerated the power of straightening is also regained. Further, in decapitated coleoptiles unilaterally supplied with auxin from an agar block, a curvature but no straightening takes place. Rawitscher (53) assumes this to be an indication that curvature and straightening are two different processes, influenced in different ways by the coleoptile tip.

Effects of Growth Regulators on the Geotropic Reaction

First some experiments will be reviewed, which are of great importance for the evaluation of the different effects on the reaction, as obtained after treatment with growth regulatory substances. They also help in interpreting the course of the geotropic reaction.

Anker (1) has compared the auxin concentration optimal for the magni-

tude of the geotropic reactions in decapitated *Avena* coleoptiles with that optimal for growth. Three substances were tested, IAA, indole-3-acetonitrile, and 1-naphthylacetic acid. They were all most effective in evoking geotropic reactions in concentrations lower than those giving optimal growth. Their optimal concentration was found exactly in the region where the growth rate showed the maximal sensitivity to small changes in auxin concentration. This is precisely what would be expected on the basis of the Cholodny-Went theory. This "auxin concentration rule" (2) for the geotropism of the *Avena* coleoptile, Anker assumes to be valid also for other plant organs. Anker found that the tips of the coleoptiles produced auxin in amounts providing for maximal rates of geotropic reaction and also that the concentration range was very narrow.

The auxin concentration rule is used by Anker (3) in the interpretation of the pH effects on the geotropic reaction. He has worked with decapitated coleoptiles assumed to be depleted of endogenous auxin. These were supplied with auxins from the surrounding solution. The optimal concentration of IAA for the production of geotropic curvatures was found to be 0.075 to 0.1 mg./l. at pH 7.8, and one third of this concentration at pH 4.1. At a constant IAA level the geotropic curvature was much weaker in more-acid solutions. Anker assumes that undissociated IAA molecules are absorbed more rapidly than the auxin anion. Consequently the absorption is higher in acid solutions and the internal IAA concentration becomes overoptimal for the geotropic reaction. A support for the interpretation is found in the fact that the effect of indole-3-acetonitrile, which does not dissociate, was independent of the pH value.

In experiments on the influence of growth substances on the geotropic reactions of wheat roots Rufelt (63) found that it was possible to refer the effects of different substances to separate physiological processes. IAA and indole-3-isobutyric acid affected the positive reaction. Their effects could be explained as arising from their effects on cell elongation. Coumarin (65) had no immediate effect on the positive reaction caused by cells which had already reached the elongation zone. At the time when cells, formed in the coumarin solution, had reached this zone an effect could be recorded both on geotropism and elongation. Thus coumarin was found to have a delayed effect on growth. The cells were attacked in a very early stage of development but the result of the attack did not appear until they began to elongate. Thus the negative reaction might be elicited by the accumulation of a coumarin-like substance in the upper part of the root. α-Phenylisobutyric acid, known as a growth promotor of wheat roots, was found to diminish the negative reactions exclusively. The conclusion must be that root growth can be affected by growth substances in at least two different ways: (*a*) by a direct effect on the elongation in progress and (*b*) by an effect on very young cells whose elongations are later inhibited or stimulated. A stimulation evoked in one part of a root can be masked by an inhibition in another part and vice versa.

Results similar to those obtained with wheat roots were found in experiments with flax roots and it is possible that roots of all species might be affected in the same way.

A third point to be remembered when comparing the effects of hormones on growth and on geotropism concerns the very great differences in the experimental times used in studying growth and geotropism. Certainly a geotropic reaction is caused by a difference in growth between the upper and the lower side of a plant organ, but the differences can be recorded with very short intervals. After half an hour considerable changes in a curvature can be observed, while in straight growth tests changes are only rarely measured at intervals shorter than 24 hr. It is a well known fact that adaptation phenomena can take place more rapidly. In determinations of freely extractable IAA in wheat roots, Rufelt & Fransson (67) found that an increase in the pH value in the root medium was followed by an increase in the IAA content of the root. The increase could be traced 1 hr. after the change in the medium and was greatest after 6 hr. of treatment. After 12 hr. in a solution with a high pH value the IAA increase was diminished and in the 24 hr. experiments the original IAA level was restored. The experiments indicate that an adaptation begins in the first 12 hr. of treatment. Physiologically an "adaptation" can be observed much earlier. The regeneration of a physiological tip in decapitated *Avena* coleoptiles is well established 150 min. after the decapitation and may be considered an adaptation phenomenon. The conclusion must be that effects of growth substances obtained in growth tests and in geotropic curvature tests can be compared only with great caution.

A fourth possible cause of false conclusions will be discussed. A geotropic reaction starts in the tip of an organ and the cells in this part of the organ have not yet reached the stage of rapid elongation. The growth rate of the organ as a whole, however, must be determined mainly by cells that have reached this stage of development and these are more distally localized. For wheat roots the following calculation can be performed. The curvatures begin 1 to 1.5 mm. from the tip. Hejnowicz (31) has studied the growth rate of wheat roots within the elongation zone, which is 4 to 5 mm. long. In the zone 0.2 to 0.8 mm. from the tip, elongation is constant and very slow but increases with the distance from the tip. It is most rapid in the oldest cells of the elongation zone. Burström (23) also claims that the most rapid elongation takes place just before the process ceases abruptly and he has found (22) that elongation of the epidermal cells of wheat roots continues for 13 hr. at 25°C. At the same temperature the geotropic reaction begins within half an hour after the geotropic stimulus commences. We are able to conclude that a change of the growth in the cells giving rise to a geotropic reaction in the tip can be completely masked by changes in elongation taking place in older cells.

Finally it should be remembered that Overbeck (50) has shown that the first stages of the geotropic reaction can be partly straightened by means of

plasmolysis. The curvature consequently is reversible to a certain extent and is not a pure growth reaction. We assume that the growth curvature is produced by auxin but we do not know whether the reversible component is also caused by auxin or evoked in quite a different way. This complication has only rarely been considered in the discussion of geotropism but it implies that effects of growth regulators on elongation and the geotropic reaction cannot be compared directly.

Brumfield (21) found the growth of *Phleum* roots markedly influenced by IAA while the amount of geotropic curvature per unit of growth was not affected. Therefore, IAA had no effect on geotropism other than that attributable to the inhibition of cell elongation. Rufelt (63) found that IAA evoked a depression of the geotropic curvature of wheat roots. This effect also could be attributed to an inhibition of cell elongation. IAA further prolonged the presentation time and the reaction time of roots of flax (59) and wheat. It was postulated that a geotropic reaction cannot begin until a threshold value is established in the quotient between the auxin content in the lower and the upper side of the horizontal root. An external supply of auxin was assumed to increase the internal concentration in the whole root. A curvature can then be produced only by a difference in auxin between the upper and the lower side greater than that necessary for the production of a curvature in an untreated root. A greater difference could be evoked during a longer presentation time. Thus we may have a parallel to Weber's law as applied to chemotaxis, because it would be possible to describe the effect of auxin as a chemical stimulus upon the cells in the elongation zone. The explanation implies that IAA affects the geotropic reaction but not the perception.

The positive geotropic reaction of wheat roots could be inverted by indole-3-isobutyric acid (IIBA). This substance is a growth promotor of wheat roots, 10^{-5} *M* being the optimum concentration. At lower concentrations, the positive curvature was gradually decreased. In indole-3-isobutyric acid at 10^{-5} *M* some roots curved slightly downwards, other roots curved slightly upwards while still others did not curve at all. In solutions more concentrated than 10^{-5} *M* the roots curved upwards. In solutions just capable of evoking upward curvatures they started at the oldest part of the elongation zone and only after a long time. In more concentrated solutions they began at the extreme root tip and the reaction time was of about the same length as that of the positive reaction. Thus the IIBA effects were found to agree with those expected to be evoked by a true antagonist to the endogenous auxin. In root and shoot elongation tests indole-3-isobutyric acid has been found not to be a true antagonist to IAA, but the discrepancy may be caused by the differences in duration of the experiments.

Audus & Brownbridge (6) found that growth-inhibiting solutions of IAA and 2,4-D reduced the rates of curvature and increased the reaction time of pea roots. Growth rates of the upper and the lower sides of the roots were both inhibited during the reaction. The authors suggested that "the same

relationship exists between growth rate and endogenous auxin concentration in the entire root as is found between growth rate and applied auxin for root segments excised from the extending zone." On this basis they calculated the amount of endogenous auxin which would be responsible for the curvatures obtained. They found the growth inhibitions too great to be caused by a redistribution of auxin in the root, and concluded that the reaction cannot be produced by IAA or any related substance. However, the authors compare the growth rate of the cells in the extension zone with that of the cells involved in the geotropic reaction. The maximum curvature had developed in 2 hr. and in this time it can hardly have extended to the most rapidly growing part of the root. Consequently the comparison may be misleading.

In low concentrations of IAA and 2,4-D, found to be growth stimulating in straight-growth tests, the rate of geotropic curvature was increased and the reaction time was shortened. The "anti-auxin" α-(1-napthylmethylsulphide)propionic acid (NMSP) (7) reduced the rate of curvature in growth promoting as well as in growth inhibiting concentrations. Reaction time was lengthened in all concentrations. A mutual antagonism was found in mixtures of growth stimulating concentrations of NMSP and growth inhibiting concentrations of IAA and 2,4-D, and the rate of curvature was higher in the mixed solution than in solutions of each component. The decreased rate of curvature elicited by NMSP is not due to a uniform growth inhibition of both sides of the root but to a partial removal of the growth inhibitions that cause the geotropic reaction. Criticism of the interpretations of Audus & Brownbridge (6, 7) has been put forward by Larsen (43) and will not be repeated here.

Ching *et al.* (24) report a direct correlation of IAA and 2,4-dichlorophenoxyisobutyric acid both on growth and on geotropic curvature of corn roots. Keitt (37) found elongation and curvature of wheat roots inhibited to about the same degree by one group of growth regulators (e.g., IAA, 1-naphthylacetic acid, and 2,4-D), curvature inhibited more than elongation by another group (e.g., 2,3,5-triiodobenzoic acid), and elongation inhibited more than curvature by one substance, isopropyl-*n*-phenylcarbamate. 2,3,6-Trichlorobenzoic acid gave two types of responses depending on the equilibration time before the initiation of the stimulus.

The influence of auxins on the geotropism of shoots is more difficult to investigate as shoots cannot be omnilaterally treated with the substances. Investigators have supplied the agents to cut surfaces of decapitated organs or via the root system. The experiments of Anker have already been mentioned. Roberts (58) succeeded in evoking positive geotropic reactions in shoots of rice seedlings by treating the roots with IAA, 2-naphthyloxyacetic acid, naphthalene-1-acetic acid, and 2,4,5-T, MIBC ester (the methyl *iso* butylcarbonyl ester of 2,4,5-trichlorophenoxyacetic acid), and achieved negative geotropic curvatures in roots by treatment with 1-naphthoxyacetic acid. However, this substance inhibited root growth. Roberts thinks the results support the Cholodny-Went theory. Maeda (45) tested the auxins

indole-3-butyric acid and 1-naphthylacetic acid, 2,4-D, and the "antiauxins" 2,3,5-tri-iodobenzoic acid and 2,4-dichloroanisole for their effect on the geotropic curvature of the leaf sheath region in wheat. He found that the curvature decreased whether a growth stimulation or a growth inhibition was obtained. This is what would be expected according to the auxin concentration rule. Schrank (68) has reported differential effects of 2,3,6-trichlorobenzoic acid on growth and geotropic curvature of *Avena* coleoptiles. With respect to the complex nature of the geotropic reaction demonstrated by the above discussion it is not surprising that different growth regulators have quite different effects on growth and geotropism. Longitudinal growth must be regarded as a sum of many different processes, which probably in the future can be partly separated by records of the different stages in the geotropic reaction chain.

The ageotropism evoked by the derivatives of phthalamic acid seems to be a separate problem. The most active representative of this group of substances is *n*-1-naphthylphthalamic acid. Ching *et al.* (24) found no correlation between straight growth and geotropic curvature in shoots and roots of corn and pea, and *Avena* coleoptiles treated with this compound. Teas & Sheehan (70) report that the negative geotropic reaction of snapdragon is inhibited by it, but gibberellic acid and IAA were partly effective in overcoming the inhibition. De Boois *et al.* (13) studied the interaction of *n*-1-naphthylphthalamic acid and IAA on growth and geotropic curvature of decapitated *Avena* coleoptiles by the method used by Anker. They found that the growth of the decapitated coleoptiles, interpreted as being caused by a residual auxin content, was not affected by *n*-1-naphthylphthalamic acid. The very small geotropic curvature, interpreted in the same way, was inhibited. Extra growth and geotropic reaction evoked by suboptimal amounts of applied auxin were inhibited; growth and geotropism evoked by supra-optimal auxin were stimulated by low concentrations of *n*-1-naphthylphthalamic acid but inhibited by higher. The results are found to co-ordinate with the hypothesis of Morgan & Söding (48) that the compound inhibits the transport of auxin through the coleoptile. Auxin within the cell would not be counteracted, only auxin transport from cell to cell. However, as the differences in growth and curvature are extremely small the results need confirmation. In one case the growth difference measured can be estimated to have been about 0.05 mm., in another the curvature was about half of one degree. The values are given only as percentages of control.

Stimulation (Susception + Perception)

The classic statolith theory is still discussed in several papers. Audus & Brownbridge (7) assume the starch-grain statoliths to be the acceptors of the gravitational force. The stimulation would arise as a direct effect of the interaction of starch grains and protoplasm along the lower walls of the sensitive cells and might consist in the synthesis or release of an enzyme. It

is further postulated that the highest sensitivity resides in the cytoplasm along the outer tangential walls of the horizontal root. The lower part of the root would thus acquire a higher concentration of the enzyme than the upper part and thereby, according to the ideas of the authors, a higher concentration of a specific growth inhibitor.

In an attempt to demonstrate the function of the starch grains as the connecting link between gravitational force and geotropic stimulation, Audus (5) tried another way of displacing the starch grains. As organic substances normally are weakly diamagnetic he suggested that starch grains might be displaced in a strong magnetic field. He found that roots of ***Lepidium sativum***, placed in a magnetic field, showed marked growth curvatures after a reaction time of 30 to 40 min.[4] The geotropic reaction of this species takes place after 4 to 8 min. The distribution of the starch grains in the cells was microscopically recorded in longitudinal sections of the root. Audus was unable to find a significant difference between the convex and the concave sides of the cells but he found a tendency that was not significant. He concludes that the experiments do not yet constitute a proof of the participation of the starch grains in the susception of gravity. An argument against this concept was presented by von Bismarck (10), who found that plants of *Sphagnum*, free from starch-grains, curve upwards when geotropically stimulated.

Hertz & Grahm (35) suggest that the statoliths may be sparingly soluble crystals or other bodies with similar properties. They found that when crystals of $CaCO_3$ were placed on a horizontal ion exchange membrane, surrounded by a salt solution, an electric potential difference was established across the membrane. The lower side of the membrane became positive. The authors found that the potential difference was caused by an exchange of the Ca ions of the carbonate crystals for K ions of the membrane. The increase in the concentration of Ca ions on the upper surface of the membrane produces a Donnan potential across the membrane. They were able to demonstrate the potential difference using *Vallisneria* leaves as membranes. The authors suggest that solid bodies within the cells, such as mitochondria, may produce the geoelectric potential difference arising in various plant organs after geotropic stimulation. The suggestion seems to the reviewer to be extremely interesting especially as Ziegler (74) has demonstrated that some cell organelle is actually displaced in a gravitational field.

In a recent report Hertz (34) has claimed that no geoelectric effect is obtained in *Avena* coleoptiles stimulated in a nitrogen atmosphere. This remarkable result indicates that metabolic processes are involved in the initiation of the potential difference, and Hertz regards the result as a support for the hypothesis that mitochondria are the functioning statoliths. According to Hertz (33) the geoelectric voltage does not appear in *Avena*

[4] The phenomenon was described simultaneously or somewhat earlier by two Russian investigators (38).

coleoptiles in darkness until 15 min. after tilting the plant organ into the horizontal position. His results are not in accordance with earlier measurements made with electrodes touching the surface of the coleoptiles. We find that the different methods of measurement used in these investigations lead to different results. It seems undisputable that a geoelectric effect really *is* produced, but at present no definite conclusions can be drawn as to its role as a connecting link between stimulus and reaction. Readers are referred to the papers by Brauner (18) and by Hertz (33).

The statolith theory in a modified form has been put forward by Larsen (42, 43) who suggests that the statoliths are not bodies capable of free translocation in the cell; instead, he visualizes the statoliths as pendulums. They may be macromolecules or larger particles fixed at one point and carrying an electric charge at the freely swinging end. The state of equilibrium of the pendulum is determined not only by the direction of the gravitational force but also by the interaction between the longitudinal electric potential of the root and the charge carried by the statolith. Thus the angle between the organ axis and the pendulum, and not the angle between the organ and the plumb line, determines the intensity of the stimulation. Larsen further assumes that the movement of the pendulums depends on the mechanical resistance in the protoplasm, and that the attainment of the state of equilibrium thus takes some time. The longitudinal potential of the plant organ would then explain the effects of the longitudinal force (the longitudinal component of gravity when it is resolved into two components acting in the transverse and the longitudinal direction of a plant organ). The longitudinal component is assumed to be of electrostatic nature but Larsen suggests that it may also be due to surface forces or an anisotropy of the cytoplasm. However, it is assumed to have an effect that is constant and consequently independent of the intensity of the stimulus. This assumption is in accord with the experimental results of Reiss (57) but is contrary to the assumptions of earlier investigators who formulated mathematical expressions for the connection between the intensities of stimulus and reaction. The hypothesis of Larsen implies that the effect of the longitudinal force is an effect on the perception. This does not agree with the view accepted earlier that the action of the longitudinal force is exerted exclusively on the geotropic reaction.

Indirectly the unilateral distribution of auxin following a geotropic stimulation has been confirmed by some investigators. Bode (12) found potassium ions to be accumulated in the lower half of *Helianthus* hypocotyls. More ash constituents, including calcium, were found in the upper half. The geotropic stimulation further increased the transport of potassium towards the base of the hypocotyl. The results were connected with earlier observations by the same author (11) that supplying IAA to the surface of tomato leaves increases their potassium content. Boysen-Jensen (15) observed an anatomical difference between the upper and the lower sides of *Phaseolus* epicotyls fixed in the horizontal position in a glass tube. The cells in the upper half became small and thick-walled and the cells in the lower half

large and thin-walled. By covering the upper side with an IAA paste, formation of the thickenings was prevented. Thickenings that were present could be removed by IAA. When epicotyls after 24 hr. in the horizontal position were turned 180 degrees, the thickenings formed on the lower side disappeared during the following 24 hr. Henckel (32) found a higher content of auxin on the convex side of geotropically stimulated roots and shoots. These results, contradictory to the Cholodny-Went theory on geotropism in roots, were obtained in a histo-chemical investigation. The method used however, is not described in detail.

The unilateral distribution of the growth regulator in the geotropically stimulated organ is commonly assumed to become established in one of the two following ways: (*a*) by a displacement, or (*b*) by a unilateral production of the growth regulator. Both possibilities have had advocates and opponents. Experiments with radioactive IAA have failed to support the hypothesis of a unilateral transport. Reisener (55) and Reisener & Simon (56) have used *Avena* coleoptiles while Ching & Fang (25) have used roots and shoots of corn, lima bean, and Alaska pea. In no case could significant differences between the radioactivity of the upper and lower sides be demonstrated.

Anker's (1, 2, 3) interpretation of his experiments with decapitated *Avena* coleoptiles are quite contradictory to these results. Decapitated coleoptiles give only negligible geotropic curvarures when kept horizontal for 75 min. However they regenerate a "physiological tip" within $2\frac{1}{2}$ to 3 hr. after the decapitation, and after that they are capable of geotropic reaction. When they are supplied with auxin from a solution surrounding the coleoptile, the geotropic reactivity is recovered much sooner. Anker has recorded the curvature, obtained after 75 min., in solutions of indole-3-acetonitrile, IAA, and 1-naphthylacetic acid. For each of these auxins, he further determined the concentration that would allow a curvature equal to that of non-decapitated coleoptiles. Indole-3-acetonitrile was found to be the most active, and in a 1.1×10^{-7} *M* solution the curvature was about 100 per cent of the controls. In IAA solutions the recovery was absolute in 5.7×10^{-7} *M* while only half the curvature of the control could be reached in 1-naphthylacetic acid solutions. The greater activity of indole-3-acetonitrile as compared to IAA was attributed to a more rapid uptake of indole-3-acetonitrile followed by its conversion to IAA within the cells. In supraoptimum concentrations, the negative geotropic curvatures were reduced, and in very high concentrations positive curvatures developed in some coleoptiles. The results were explained in terms of the Cholodny-Went theory and it was assumed that the growth substance absorbed via the apical cut surface was translocated to the lower side of the coleoptile.

Brauner & Hager (20) have suggested that not auxin but a cofactor to auxin would be translocated. They have used two methods for a separation of the geotropic stimulation from the reaction in hypocotyls of *Helianthus*. First they stimulated intact seedlings at 4°C. Under these conditions no reaction takes place. Returned to the vertical position and warmed to 20°C.,

the hypocotyls curve according to the stimulus. Second, decapitated plants were placed in a darkroom at 20°C. for four days. After that a geotropic stimulus gave no reaction. Curvatures were formed, however, when IAA was applied through a glass tube to the cut surface of such hypocotyls. Brauner and Hager, therefore, assume that the inability of the plants to produce a curvature is caused by auxin depletion. The fact that a geotropic perception can take place without a reaction makes Brauner and Hager suggest that the stimulation takes place in the following steps. Under the influence of the gravitational force, the easily diffusible cofactor is translocated toward the lower side of the organ. After that, a period of metabolic binding of this substance sets in at the places where it is accumulated. Oxygen is consumed in the binding process, and no geotropic reaction was obtained after stimulation in a nitrogen atmosphere. The cofactor then combines with auxin to form a complex active in stimulating growth. Thus the cofactor is translocated during the stimulation of auxin-free hypocotyls, but a growth reaction takes place only after the addition of IAA.

Contradictory to the opinion of Brauner and Hager about geotropic perception, De Wit has used the phrase "without growth substance no geotropism." His statement is founded on the following experiments. Decapitated coleoptiles, at first kept horizontally in water and then vertically in a growth substance solution, did not curve geotropically. Decapitated coleoptiles, kept horizontally in water for periods up to 90 min. and then in the same position in a growth substance solution, showed no change either in the receptivity or in the reactivity as a result of the treatments. In 1923 Brauner found that geotropic perception is possible in decapitated coleoptiles in the absence of auxin, and Brauner & Appel (19) have recently reported that experiments confirming this opinion have been performed and will soon be published.

It seems to the reviewer that the experiments of Brauner and Hager as well as those of Anker's school should be discussed in connection with the experiments of Guttenberg & Büchsel (28). According to these authors, IAA and a diffusate from coleoptile tips, laterally applied to intact *Avena* coleoptiles, had identical effects. Applied to "auxin-free" coleoptile cylinders, however, the diffusate was found to have an immediate effect while the effect of IAA was delayed for 3 hr. The curvature produced by the IAA was, however, larger than that produced by the diffusate. In order to get "auxin-free" cylinders, coleoptiles were excised from the seeds and decapitated twice, the second decapitation being performed 4 hr. after the first. "Auxin-free" cylinders attached to the mesocotyl stumps regained their capacity to respond immediately to applied IAA. *Helianthus* hypocotyls behaved in the same way qualitatively, but the time delay after application of IAA was longer and the curvature beyond the control value greater than that shown by the coleoptiles. The hypocotyls were decapitated 36 hr. before the experiment. In the *Helianthus* hypocotyls it was further observed that the increase in growth produced by IAA extended both upwards and downwards, thus

indicating a non-polar migration of the active material. The conclusion drawn by the authors was that IAA does not increase growth directly but acts through an activation of an auxin precursor. Auxin, at that time, meant the auxins *a* and *b* of Kögl.

What really happens is not easy to say and we do not know the mechanism behind the regeneration of a physiological tip. It is obvious, however, that the result of a decapitation is not only a depletion of auxin. This is also obvious from the experiments of Brauner and Hager. The "auxin-free" hypocotyls had to be stimulated for 4 to 5 hr. before a reaction was produced, but intact hypocotyls developed a curvature after 10 min. of stimulation. Further, the reaction of intact hypocotyls reached the greatest angle after 2 hr. while the "auxin-free" hypocotyls needed 4 hr. Brauner and Hager recorded the geotropic reaction induced by a temporary gravitational stimulation. A curvature in the negative direction was always produced. In the series with cooled hypocotyls this was followed by a straightening and a curvature in the opposite direction. In decapitated plants only the first curvature appeared, and this was not straightened even when IAA was added. Identical results were obtained by de Wit (73) using *Avena* coleoptiles. Thus there is a fundamental difference in the mode of reaction of intact and decapitated plants.

The second curvature is assumed by Brauner and Hager to be produced as a result of the stimulus received during the first curvature when the hypocotyls deviate from the vertical position. The absence of the counter-reaction in the decapitated coleoptiles might be caused by a deficiency in the cofactor. As for auxin, it is suggested that the cofactor is produced by the apical parts of the plants, and thus its center of production is removed by decapitation. Now it can be argued that if the cofactor is more easily diffusible than the auxin, the former would be assumed to disappear from the stem stump at least as fast as the latter. Otherwise we must assume that the experiments have been performed just at the moment when there is an amount of cofactor exactly sufficient for the first reaction to take place, but not for the second. It would then be interesting to test the stem stumps somewhat earlier and somewhat later than at the time chosen by the authors.

The contradiction between the interpretations of Anker and the results of experiments with radioactive IAA might be explained by the suggestion of Brauner and Hager that a cofactor is displaced. Another possible explanation is that the treatment accelerates the regeneration of the physiological tip. De Wit has denied this latter possibility for two reasons. He refers to Gorter (27) as having shown that no regeneration of a physiological tip takes place when decapitated coleoptiles are supplied with IAA. The reviewer has been unable to find this statement in Gorter's paper, and the reference may be the result of some misunderstanding since IAA was not known as a growth regulator at the time. Secondly, in his own experiments, de Wit found that the geotropic curvature in IAA solution proceeded at a rate that remained constant for several hours. He expected that an increase in the auxin content

during the geotropic curvature, caused by a regeneration of a physiological tip, would change the rate of bending. However, this expectation is founded upon the assumption that the externally supplied auxin really induces the curvature. If the whole curvature is caused by endogenous auxin produced by the new physiological tip no change can be expected. Summing up, it seems to the reviewer that we have at present so many contradictory results that no definite interpretation is possible.

In a recent paper, Brauner & Appel (19) presented some experiments which, according to the authors, demonstrate the lateral transport of a growth regulator. They used a method introduced by Boysen-Jensen in studies on phototropism, and inserted mica-plates in the median plane of tips of *Avena* coleoptiles. In all their experiments the plane including the two vascular bundles was vertical during the geotropic stimulation. The mica plates were placed either in the same plane or perpendicular to it. In the latter case the mica plate impedes a possible transport of auxin to the lower side of the coleoptile during stimulation. The curvatures were always greater when such a transport was not hindered, and the authors conclude that the experiments prove the existence of a lateral transport. In order to test the possibility that the differences in curvature might be caused by having the mica plate in one of the series inserted in the vascular bundles and in the other series between the bundles, the longitudinal growth was determined in coleoptiles with mica plates inserted in the two different planes. Growth was always more inhibited in coleoptiles with the mica plates through the vascular bundles. Consequently, the coleoptiles producing the greatest curvatures were most inhibited. The conclusion from this result that the difference in curvature would be greater without the wound effect is not convincing since it is founded on the assumption that the faster the growth, the greater the curvature.

In a third series of experiments the amount of auxin diffusing in the lateral direction within the coleoptile tip was determined. The coleoptiles were dissected in two halves by means of a vertical incision, 2 mm. deep, between the vascular bundles. One of the halves was removed by a horizontal incision and an agar block was placed in the corner formed by the incisions. The amount of growth substance obtained in the agar block was determined in the *Avena* curvature test. From coleoptiles standing vertically, only negligible amounts were obtained, but when the coleoptiles were placed horizontally with the agar block downwards, the substance collected was able to produce a curvature of about 3 degrees more than the controls. A curvature of about the same magnitude was obtained by 10^{-7} *M* IAA in the agar block. It seems to the reviewer that a determination of the amount of auxin obtained in similarly incised coleoptiles but with the agar block on the cut surface facing upwards is desirable. According to Audus & Brownbridge (6) and Bennet-Clark *et al.* (9) the geotropic stimulation is assumed to induce a production or a release of a growth substance. If this is true the increase in auxin might be explained without assuming unilateral transport.

Audus & Brownbridge (6) suggest that the geotropic reaction of pea roots is induced by a *de novo* production of some growth inhibitor. The inhibitor

is thought not to be IAA, nor even related to this substance. The experiments on which this conclusion is based have already been discussed. The main argument against the concept of a lateral displacement seems to be that there is a total decrease in growth rate during the geotropic reaction. A similar decrease was also found by Bennet-Clark *et al.* (9) and Rufelt (60). If the suggestion of Anker concerning coleoptiles is assumed to be valid for roots, i.e., if the tip produces auxin in amounts that provide for a maximal rate of geotropic curvature, the decrease is an argument against the concept of a displacement. Larsen (39) and Bara (8) have also published results indicating that the geotropic curvature of roots is accompanied by a decrease in growth.

As to stems, the results of Maeda (45) indicate that the negative geotropic reaction of the intercalary growth region of stem segments of wheat is produced by an unequal production of auxin. Arslan & Bennet-Clark (4) found that the geotropic bending of grass nodes is caused by an increase in growth that would never take place in unstimulated nodes. Bara (8) determined the optimum auxin concentration for phototropic and geotropic curvatures in decapitated *Helianthus* hypocotyls and *Avena* coleoptiles. The substance was supplied to the plants via a glass tube connected to the cut surface. The optimum concentrations for phototropic curvatures were IAA at 10^{-6} *M* for hypocotyls and 10^{-7} *M* for coleoptiles. For geotropic reactions, the optima were 10^{-8} *M* and 10^{-10} *M* respectively. Bara assumes that the geotropic reaction is produced by an increase in the production of auxin on the lower side of the organ and that the auxin production is totally increased. Phototropic reaction is produced by a decrease in the auxin content on the illuminated side with a decrease in the total auxin content. Bara finds support for his interpretation of geotropism in experiments on the influence of gravity on the growth of shoots and roots. When rotated on a klinostat, shoots increase and roots decrease their growth. The results are interpreted as arising from a stimulation of the production of auxin caused by gravity. As to the roots, the results agree with those of Larsen (39).

Bennet-Clark *et al.* (9) have suggested that the increase of growth inhibitor in the lower half of a geotropically stimulated root is not the result of a stimulation of the production but of a release of a substance, probably from a "vacuolar stock." The act of turning the root is the cause of the release, and the turn promotes "quite abnormal chemical or physicochemical changes, just as turning a cupboard full of chemical reagents on its side suddenly might well provoke chemical reactions." It may be argued that rotation of the cupboard on a klinostat would provoke chemical reactions as well, but according to the experiments of Larsen rotation of roots produces quite regular changes of growth with the magnitude depending on the rate of rotation. Another phenomenon not in accordance with the hypothesis concerns the turning of a plagiotropic root from a position of stimulation to the vertical position, the tip pointing downwards; this is followed by a negative curvature. For a more detailed description of these phenomena see Rufelt (66).

Rufelt (63, 64) has suggested that a geotropic stimulus induces an increase

in the production of auxin on the lower side of a root, this increase being the cause of the positive geotropic reaction. It is suggested that the negative reaction is produced by an increase of another growth inhibiting substance on the upper side. The geoelectric effect is considered the cause of this increase. A shift in the positive direction on the lower side involves a rise in the pH value, which has been shown to increase the auxin content of wheat roots (67). On the upper side, a shift in the negative direction induces an increase in the production of the substance causing the negative reaction. The negative reaction is shown to be most pronounced in a solution with a low pH value and is almost absent when the pH value is high. The inhibitor causing the negative curvature is assumed to be easily oxidizable and this would explain the transient nature of the negative curvature. The results of Metzner (46) should be considered in this connection. He treated geotropically stimulated roots with a solution of a silver ammonia complex and found that more silver was precipitated in the upper half than in the lower, indicating that the reduction-oxidation potential is lower in the upper part.

Other Observations

Brain (16) determined the percentage of *Lupinus polyphyllus* seedlings giving a geotropic reaction after 20 min. of stimulation. She found that more curvatures were formed during experiments performed in summer than in winter. A closer analysis indicated that the differences were due to differing amounts of illumination. Pilet (52) found that illumination prolonged the presentation time and reaction time of *Phycomyces*. Mohr & Pichler (47) observed that seedlings of *Sinapis alba* grew more nearly vertical after exposure to red light. They suggested that the negative reactivity (Reaktionsfähigkeit) was increased. Teas & Holmsen (71) reported that the geotropic reactions of corn and pea shoots were inhibited by gamma radiation. The reactivity could not be restored by supply of IAA or 1-naphthylacetic acid.

Halbsguth (29) found that phalloidin decreased the geoinduction in gemmae of *Marchantia polymorpha*. Phalloidin is known to disturb ATP metabolism and the effect on geotropism was assumed to be mediated by a cofactor to IAA. The observation by Reiff & von Guttenberg (54) that IAA transport is increased by ATP is interesting in this connection.

A differential effect of ether narcosis on growth and geotropism in *Avena* coleoptiles observed by Bottelier and Roosheroe was assumed to be caused by an influence on the transport of auxin.

Variations in the geotropic reactivity attributable to endogenous factors have been studied by Shibuya (69) in experiments with the antigeotropism induced by eosin in the primary (seminal) root of rice. The degree of antigeotropism varied with the amount of after-ripening of the seed, with the germination rate, with the length of the leaf in the coleoptile, with the length of the root, and with the variety. It was also found to be more pronounced in tetraploid roots than in diploid roots. Bismarck (10) found that the pseudo-

podium of *Sphagnum* was geotropically and phototropically reactive but after the opening of the capsule both kinds of reactivity were lost. Finally, the experiments of Kaldewey (36) with the hook-forming flower stalk of *Fritillaria meleagris* should be mentioned in this connection. The stalk performs movements induced by gravity. Kaldewey found that the cells in the prospective convex side of the stalk start their elongation earlier than the cells on the opposite side; however, elongation is also terminated earlier on the convex side, and therefore the hook straightens out again.

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PHOTOCHEMICAL AIR POLLUTION DAMAGE TO PLANTS[1]

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Air is a natural resource vital to man, essential for plants, and necessary for many of man's community, domestic, and industrial activities. Air is a mixture of gases and is normally colorless, odorless, and tasteless. The chemical composition of air varies but is essentially composed of 78 per cent nitrogen, 21 per cent oxygen, with argon and other rare gases, carbon dioxide, and water vapor making up the balance. The quality of air varies for a number of reasons but principally because of emission contaminants arising from economic and social developments throughout the world. Air pollution results from this contamination of the atmosphere. The common manifestations of air pollution resulting from man's contamination of the atmosphere are: interference with visibility, damage to property, irritation to the senses, adverse effects upon the public health and welfare, and damage to vegetation.

Clayton *et al.* (7), Faith (18), and Mallette (32) provide accounts of the history and evaluation of air pollution. Magill *et al.* (31) and Mallette (32) discuss the problem of air pollution as related to interference with visibility, while Drinker (14, 15, 16) reports on air pollution as it affects the public health and welfare.

Air pollution damage to vegetation has been recognized for more than a century. First interests in the agricultural aspects of air pollution were largely concerned with smoke and fumes from industrial wastes, particularly those of chemical manufacture, smelting, and clay and glass production, as well as combustion effluents from burning of coal and petroleum. The principal phytotoxicants recognized several decades ago were fairly specific, simple molecules, such as ethylene, fluorides, hydrogen sulfide, and sulfur dioxide. The specific effects of these toxicants on plants have been well described and figured. Information published by Middleton *et al.* (38), Thomas (52), Thomas & Hendricks (53), and Zimmerman (62) permits identification of the toxicant responsible for plant damage based on typical symptom expression.

In the last decade a new group of air-borne phytotoxicants were described and have been identified as components of smog. Since "smog" refers to smoke and fog, neither of which are responsible for vegetation damage, the polluted air mass containing the damaging incitants is herein called "photochemical" or "community" air pollution. The toxic components in community air pollution are typically the oxidation products of hydrocarbons and result either from the dark reaction of ozone and olefins or the photolytic reaction of nitrogen oxides and hydrocarbons in the presence of sunlight. It

[1] The survey of literature pertaining to this review was concluded in January 1961.

is the purpose of this paper to describe the occurrence and distribution of photochemical air pollution, enumerate some of the raw materials and products of these oxidation systems responsible for plant damage, and indicate the biochemical, physiological, and pathological effects of these contaminants upon plants.

Oxidant

Damage to leafy vegetable and field crops of a type quite different from that resulting from exposure to sulfur dioxide, halogens, and a number of other air pollutants was first observed by Middleton *et al.* (42) in southern California in the Los Angeles area in 1944. This unique and typical injury consisted of a silvering, glazing, bronzing, and sometimes necrosis of the lower leaf surface of sensitive dicotyledonous plant species. Concomitant upper leaf surface injury occurred only after the lower leaf surface had been extensively damaged except in those plants in which stomates were located freely in the upper leaf surface. In this case upper leaf surface injury sometimes occurred in the absence of lower leaf surface injury. Although this type of injury is commonly called "smog" damage, it is correctly described now as "oxidant" damage. The unique nature of oxidant injury found in urban areas provided a means of identifying the toxicants responsible and evaluating their effects upon plants.

Ozone-olefin dark reactions.—It was through the cooperative study of the California Institute of Technology, the Los Angeles Air Pollution Control District, and the University of California that Haagen-Smit *et al.* (21) demonstrated for the first time that ozonated olefins were capable of causing oxidant injury to sensitive, laboratory grown, indicator plants that was indistinguishable from the injury observed on field grown plants in the Los Angeles basin. They showed that oxidant damage was reproduced by exposing plants to a mixture of ozone and a variety of olefin vapors when reacted at high concentrations and diluted in an airstream. Leaf symptoms produced by ozone and butadiene, 1-pentene, 1-hexene, cyclohexene, 3-heptene, 1-octene, 4-nonene, benzene, and gasoline, all resembled those that occurred naturally. They further demonstrated that ozone alone and the hydrocarbons alone, as well as the end products of the ozone-olefin reaction, namely, organic acids and aldehydes, were not able to produce the unique and typical oxidant silvering. They suggested that the toxicant must be an intermediate, transient material, possibly an ozonide or a related peroxide.

Mader *et al.* (30) reported that formic acid, present in Los Angeles air in amounts up to 0.4 p.p.m., was one of the most concentrated of several organic acids found in plant leaves. They also determined that leaves exposed to ambient oxidant or plants exposed to mixtures of ozone and hydrocarbons contained almost twice the amount of formic acid found in untreated plants. Acetic and lactic acids were also present in exposed plants and at levels above those in unexposed ones but in quantities considerably less than formic acid. They suggest that formic acid may be responsible for the leaf glazing despite

the fact that no evidence is given from direct fumigations to support this thesis. Middleton *et al.* (38) report that the fumigation of oxidant-sensitive indicator plants with 8 p.p.m. of formic acid in a dynamic flow system failed to produce any oxidant injury.

The work of Haagen-Smit and his associates provided a basis for others to attempt the identification of the specific product or products responsible for oxidant injury. Darley *et al.* (13) undertook a study to determine the structure of the phytotoxicant, its mode of formation, and its properties, utilizing long-path infrared spectroscopy in conjunction with the response of plants to ozone-olefin reactions. They demonstrated that the reaction of ozone with 5, 6, and 7 carbon olefins is quite rapid in the gas phase at low concentrations and that the reaction mixtures produced a toxic material that caused typical oxidant-type air pollution injury. Whereas Haagen-Smit and associates demonstrated that ozonated 1-pentene produced a strongly active phytotoxicant and that ozonated 2-pentene products containing 2 and 3 carbon atoms failed to produce an active phytotoxicant, Darley and colleagues demonstrated that oxidant injury could be produced through the ozonation of 2-pentene when reaction times were properly matched. This suggests that concentration and reaction time limited the formation of the phytotoxicant and that toxicity is not necessarily solely a function of carbon atoms in the molecule. Darley *et al.* (13) prepared ozonides from 1-pentene and 2-pentene and fumigated sensitive indicator plants without producing any injury after about a 2 hr. exposure at approximately 4 p.p.m. The ozonides produced were rather stable species whereas the phytotoxicant resulting from the ozonation of olefins was obviously an unstable species with a half-life of no more than a few minutes. These results suggest that either a transitory ozone-olefin complex or an unstable zwitter ion formed by the decomposition of the olefin complex is perhaps the actual toxic substance. Attempts to confirm this view by scavenging the toxicant with excess aldehyde or sulfur dioxide were unsuccessful. Arnold (1) demonstrated that the amount of chlorosis induced in duckweed resulting from exposure to ozonated 3-heptene and 2-pentene was a satisfactory quantitative measure of damage. The results of his experiments suggest that decay of the phytotoxicant follows a first order or a pseudo-first order reaction, and further, that the half life of the phytotoxicant from ozonated 3-heptene is about 15 min. while that of ozonated 2-pentene is about 3 min. He suggested that the difference in stability of the two phytotoxicants may be explained in terms of the zwitter ion mechanism as advanced by Criegee (8, 9).

Further studies by Darley *et al.* (12) on the phytotoxicity of gaseous ozone-hydrocarbon reaction mixtures have shown that oxidant plant damage was obtained from all reactions whose products after cleavage at the double bond contained three or more carbon atoms. Ozone reactions with propylene and both *cis* and *trans* 2-butene produced no phytotoxicant. This work reports for the first time damage from ozone-olefin mixtures of the following hydrocarbons: 3-methyl-1-butene, cis-3-methyl-2-petene, cis- and trans-3-

hexene, toluene, and cumene. The amount of injury resulting from these materials shows that the addition of a methyl group at the double bond markedly reduces plant damage, suggesting that the methyl group interferes in some way with the formation of the phytotoxicant.

Whereas the earlier work of Haagen-Smit *et al.* (21) had shown that aldehydes and organic acids did not produce typical oxidant damage and that in fact, acrolein produced only atypical damage on some of the test plants used, work of Darley *et al.* (12) reported typical oxidant damage resulting from exposure of sensitive indicator plants to 2 p.p.m. of acrolein alone. While the authors state that the damage was not severe, they do report that it was definite and indistinguishable from the underleaf bronzing typical of oxidant damage. This observation difference may be due to the methods used in fumigation and dispensing of chemicals as well as ecological factors prior and subsequent to exposure.

Factors influencing susceptibility to damage.—A number of environmental factors affect the susceptibility of plants to oxidants resulting from the ozonation of olefins. Symptom expression and severity of damage are functions of plant species as well as of toxicant concentration and length of exposure. In addition to species, concentration, and dosage, Middleton (35) reports that the application of nitrogen to soil at the rate of 45 lbs. per acre increased the amount of injury to spinach leaves 40 per cent, and romaine lettuce 80 per cent, compared to plants not receiving nitrogen additions. Injury to barley was increased 10 per cent with nitrogen addition over that grown in soil without nitrogen. The addition of nitrogen to oats increased their susceptibility 20 per cent. It was also demonstrated that increasing the number of days of exposure to 55°F. decreased the sensitivity of romaine lettuce to oxidant as compared with plants exposed to the same concentration of toxicant but maintained at 75°F.

Another factor affecting oxidant symptom development besides plant species is the age of leaves. For example, Middleton *et al.* (40) showed that in controlled fumigation of tomato plants, the plant damage indices for successive leaves from base to apex were 0, 0, 1.3, 2.9, 6.5, 9.4, 5.2, 1.6, and 0. Injury indexes for successive alfalfa leaves on 15 plants from base to apex after 4 hr. of exposure were 6.8, 7.1, 6.8, 6.2, 4.5, 1.1, and 0. The injury indexes for successive leaves from base to apex of bean plants after a 3-hour fumigation were 7.8, 6.7, 6.1, 4.3, 2.5, and 0. The response of primary leaves of pinto bean from 2 to 35 days following emergence shows that the primary leaves obtained a maximum susceptibility about 2 weeks after emergence and remained equally susceptible until 5 weeks old.

Taylor *et al.* (51) corroborated the effect of reaction products of ozone and olefin upon 14-day-old pinto bean leaves and remarked that by contrast the primary leaves of 7-day-old plants and the immature trifoliate leaves of 14-day-old plants were resistant to these reaction products although they were usually very susceptible to injury from the naturally occurring oxidant.

Pathological anatomy of oxidant damage.—The details of the effect of

oxidant upon cells are well described and figured by Bobrov (3, 4, 5). Her elegant studies of living tissue have shown that the first response of cells is their engorgement, especially of the guard cells and surrounding epidermal cells. As the epidermal cells become stretched, usually through the distention of the guard cell, they frequently rupture and collapse. This observation accounts for the change from the blister-like appearance of the epidermis to that of the water-soaked appearance in which plasmolysis of cells in the spongy parenchyma occurs. The silvering and glazing of the leaf surface is attributed to the dehydration and shrinkage of many of the mesophyll cells through the formation of enlarged, air-filled, intercellular spaces. When leaves are exposed to low concentrations of oxidant for short periods of time, they frequently appear chlorotic rather than expressing the typical glazed lower surface. Bobrov (5) demonstrated that this yellowing is due to the plasmolysis of a limited number of cells, such that a number of chloroplasts are destroyed while adjacent cells may remain intact and the chloroplasts functional. According to Bobrov (3, 5), chlorotic spots on grass leaves typically appear on the upper leaf surface as a result of injury from oxidant and likewise usually appear as longitudinal bands because of the vein system, disposition of stomates, and the location of the chlorophyll-bearing cells. The location of damage on some other plants varies from the typical because of leaf anatomy and stomate distribution, e.g., endive in which no organized palisade layer exists and stomates are distributed on both leaf surfaces. In such species, chlorosis and silvering may occur indiscriminately on both leaf surfaces.

The damage patterns reported by Noble (44) certainly show that injury from oxidant occurs only on structurally and functionally developed leaves. These injury patterns observed in the field have been reproduced in laboratory fumigations. The fumigations clearly show that oxidant injury typically will first affect those leaf areas that have just begun to function actively. Oxidant injury to vegetation, therefore, may be readily recognized in that it does not affect the terminal, immature leaves nor the basal, fully matured leaves. Bobrov suggested that possibly the injury occurs on those leaves that have just become functional because of the lack of suberization of those cells and the protection of older ones by the development of suberin.

Illustrations and colored photographs of oxidant plant injury are found in publications of Middleton & Paulus (43), and Middleton *et al.* (37, 38, 42). Lists of plant species and their susceptibility to oxidant injury appear in works of Middleton & Paulus (43), Middleton *et al.* (41, 42), and Thomas & Hendricks (53).

Effect of oxidant on plant growth.—In addition to the important injury to the foliage, there are significant adverse effects upon plant growth in the absence of visible leaf injury. For example, Hull & Went (24) and Hull *et al.* (25) have shown that the diurnal fluctuation in sensitivity of the *Avena* coleoptile almost certainly results from variations in air pollution during the day and night and that alterations in curvature resulted from exposure to

oxidant. Hull & Went also demonstrated that growth of alfalfa, sugar beet, endive, spinach, and tomato was suppressed when the plants were grown in ambient air containing oxidants as compared to the same plants grown in ambient air passed through an activated carbon filter. Koritz & Went (28) showed that the fresh weight of tomato tops exposed to ozonated hexene during lighted hours was consistently less than the fresh weight of comparable control plants; the differences in dry weights were small and not significant. They were unable to show a positive correlation between degree of stomatal aperture and extent of damage. They did, however, report that conditions favoring the opening of stomata, such as light and turgor, were conditions favoring susceptibility to the phytotoxicants. While the growth of plants fumigated in the light and midday was significantly decreased by the phytotoxicant, there was no effect on tomato plant growth when fumigated in the dark or light just following a 12-hour dark period or in the dark after 1½ hours of light. Tomato plants that were given limited water supply resisted injury compared to those receiving abundant water. They also demonstrated that transpiration and water uptake rates of tomato plants were lowered by exposure to oxidant. When plants were subjected to repeated fumigation exposures, there were repeated decreases in transpiration followed by partial recovery. Bleasdale's (2) experiments, which showed reduced growth of rye grass in a sulfurous atmosphere in England, suggest that oxidant rather than some other toxicant may be responsible for growth inhibition.

Taylor *et al.* (50) have shown that tree growth likewise may be reduced as a result of exposure to the products of ozone-olefin reactions as well as naturally occurring oxidant. Avocado seedlings were fumigated with reaction products of ozone and 1-hexene for 280 hr. over a period of about 8 weeks. The investigators found that this fumigation reduced avocado stem elongation 56 per cent, leaf width 35 per cent, length 35 per cent, and stem diameter 21 per cent. The fresh weight of seedlings, including the root system, was reduced 52 per cent while the dry weight of seedlings including the root system was reduced 58 per cent; all comparisons were made with comparable lots of avocados grown in carbon-filtered air. Taylor (49) has further shown that the reaction products of ozone and 1-hexene significantly reduced the growth of rooted Lisbon lemon cuttings as well as Lisbon lemon scions grown on citrange rootstock. Similar effects were observed upon grapefruit seedlings. No visible symptoms of leaf injury were observed in either the plants exposed to reaction products or in control plants grown in activated carbon-filtered air. As the exposure of lemons to both reaction products and ambient air containing oxidants was extended, there was premature senescence and drop of the older lemon leaves. Taylor also reports that the rate of water use by lemon cuttings and lemon scions was reduced as much as 25 per cent by the reaction products of ozone and hexene; this reduction in rate of water use no doubt had a significant effect upon the reduction of dry weight of exposed plants.

Similar reduction in growth, as a result of exposure to oxidants in ambient air, has been shown by Todd *et al.* (58) to occur in Kentia palms. The alterations in growth were obvious but occurred only after an 8-month exposure, primarily because of the extremely slow growth rate normal to Kentia. Palms grown in an unfiltered glasshouse were somewhat larger and less chlorotic than those in the lathhouse, whereas the palms in the carbon-filtered glasshouse were noticeably larger and averaged one leaf more than the palms in the ordinary glasshouse. The leaves on plants grown in a carbon-filtered glasshouse also were longer and darker green in color. This experiment also points out that an exclusion of naturally occurring oxidants enables the palms to regain a healthy normal color and to grow more rapidly than those not protected with activated carbon filters.

Todd & Garber (57) studied in some detail the effect of ozonated gasoline and ozonated hexene on the growth of pinto and red kidney beans and Alaska pea. The plants were all container grown, and prior to treatment were placed in glasshouses provided with activated carbon filters. Materials were fumigated for varying lengths of time, but mostly from 26 to 42 hr. total. Frequent records were made for the purpose of learning the direct effect of periods of fumigation upon growth and reproduction. Their results corroborated those of others in showing that there were lowered growth rates in plants following fumigation with low concentrations of ozonated hexene and gasoline. The effects were to reduce growth in the absence of visible symptoms of injury. These effects were evident from measurements of linear growth, leaf area, fresh and dry weights, and fruit production. It was particularly interesting that although the fumigation had no effect upon the number of leaves initiated, the fumigation did have a very significant effect upon the enlargement of both the primary and trifoliate leaves; those leaves exposed to fumigation were significantly smaller than those not exposed. Whereas the treated leaves did not show a higher water content than did the control leaves, stems of plants so treated did show a higher water content. The height attainment of Alaska pea plants was slowed temporarily by as little as a half-hour single fumigation with ozonated hexene, although the treated plants fully recovered and grew normally one or two days later. These results suggest that the products of ozonated hexene seem to inhibit cell expansion.

Taylor (49) has shown growth suppression in Petunia and tomato. The rate of growth of Petunias was reduced about one-half over a 3-week exposure period compared with control plants grown in clean air. Leaf size was reduced by 50 per cent, and blossom initiation was completely suppressed by exposure to the reaction products of ozonated hexene. Naturally occurring oxidants caused similar growth and blossom suppression and induced typical oxidant injury on Petunia leaves. The size of tomato leaves was reduced, but the number of nodes and number of fruit spurs were not altered from those on plants grown in clean air. However, 60 per cent of the blossoms aborted

in naturally occurring oxidant-contaminated air, whereas practically all the blossoms on tomato plants grown in carbon-filtered air were normal and set fruit.

Effect of oxidant on photosynthesis and respiration.—Some of the effects of ozonated hexene on the life processes of plants have been studied by Erickson & Wedding (17) and Wedding & Erickson (59). They demonstrated that hexene alone did not have any significant effect on photosynthesis, respiration, nor on the conductivity of the water in which the duckweed plants floated during the course of exposure. They suggest that an increased chlorophyll content seemed to result from exposure to hexene alone. The chlorophyll content in plants exposed to ozonated hexene for a 24-hour period was reduced about 50 per cent compared to that of the control plants, whereas the photosynthetic rate was about 30 per cent of that of the control plants. They also demonstrated that respiration was stimulated by exposure to ozonated hexene. The increased conductivity of the water supporting the plants indicated that a 24-hour exposure destroyed the semipermeability in 30 per cent of the cells. Further studies on the effect of exposure to ozonated hexene upon permeability are reported by Wedding & Erickson (59); they show a decrease in the permeability of cells of pinto bean leaves to radioactive phosphate. The decreased permeability resulted from exposure to 3-hour fumigations; when longer exposures were made there was a reversal in the decreased permeability, enough that it increased the permeability of the leaf cells to phosphate. The increased permeability was always associated with the appearance of visible injury to the exposed leaves. They demonstrated the same effects of ozonated hexene upon permeability and water uptake through exposure of potato tuber discs. These two cellular studies suggest that the influence of reaction products upon growth suppression as measured by linear growth, fresh and dry weight, as well as by water transport and use in the absence of visible injury, may be associated with cell development and alterations in permeability.

Todd (56), in further studies on the effect of ozonated hexene upon respiration and photosynthesis, demonstrated that the respiration rate of pinto bean leaves increased more than four times over that of untreated control plants, whereas the rate of photosynthesis was less than that of controls. However, no stimulation of bean leaf respiration nor inhibition of photosynthesis occurred unless visible injury developed. He also showed that treatment with ozonated hexene in concentrations causing changes in respiration and photosynthesis in bean leaves, did not alter these processes in Valencia orange leaves. Todd (54) also demonstrated that respiration of commercially mature, picked lemon fruit is markedly stimulated by exposure to ozonated hexene at low concentrations for as little as 1 hr. Longer treatments gave a greater respiratory stimulation, as much as $3\frac{1}{2}$ times that of the control. Hexene alone had no effect on the respiration rate. The stage of ripeness had some effect on the magnitude of respiration since dark green fruit responded more than light green fruit and yellow fruit responded the least.

Nitrogen oxides-olefins photochemical reactions.—At the same time as Haagen-Smit *et al.* (21) published their pioneer work relating that oxidant damage to vegetation resulted from ozone-olefin dark reactions, they reported that similar plant injury occurred to sensitive indicator plants when they were subjected to a mixture of olefin, nitrogen dioxide, and sunlight. The photochemical reactions resulting from the oxides of nitrogen and hydrocarbons have been studied by many investigators but are especially well summarized by Haagen-Smit and by Stephens *et al.* (47). In the simplest form these reactions result in the formation of ozone and a variety of products; the amount and rate of ozone formation is a function of hydrocarbon substrate. Among the products formed are formaldehyde and acrolein, both of which have already been shown to be incapable of producing typical oxidant injury at naturally occurring concentrations, but acrolein at higher concentrations is capable of producing symptoms indistinguishable from oxidant injury. Taylor *et al.* (51) have shown that the photochemical reaction products of nitrogen dioxide and 1-hexene severely injure leaves of Petunia after the substrate has been reacted for as little as 15 min. in the presence of black light fluorescent lamps. They report that the injury from these irradiated mixtures was identical in appearance and location on the plant with that caused by naturally occurring oxidants. The amount of injury was increased when either the concentration of reactants or the intensity of the irradiation was increased. They point out that plants were not injured when exposed solely to the vapor of nitrogen dioxide or to that of 1-hexene, either with or without irradiation. Also of significance was their observation that the primary leaves of 14-day-old pinto beans, which are extremely sensitive to the dark ozone-olefin reaction products, were not injured by the products of the photochemical reaction of nitrogen dioxide and 1-hexene. The products of this reaction, however, did cause severe glazing of the lower leaf surface of the young trifoliate leaves of 14-day-old plants and the primary leaves of younger 7-day-old plants. In this case the glazing was identical and indistinguishable from that observed on plants exposed to naturally occurring oxidants.

Stephens *et al.* (47) reported that when low concentrations of simple olefins and nitrogen oxides in air are irradiated, a specific product, peroxyacetyl nitrite, is detected and is capable of isolation. Fumigations with peroxyacetyl nitrite (PAN) have shown that concentrations well below 1 p.p.m. in air produced typical oxidant damage on sensitive indicator plants. This is the first pure compound isolated from photochemical reaction mixtures that has been found to produce the characteristic oxidant damage symptoms so long observed in many communities throughout the world. This particular compound also has the distinction of being the only one so far identified that is capable of causing both oxidant damage as well as irritation to eyes. Perhaps because of the newness of the discovery, there is no information yet available on the physiological and biochemical effects of this toxicant on life processes of plants. All of the hydrocarbons used by Stephens and

associates gave typical oxidant plant damage. The severity of damage was directly related to the speed of the reaction; the greatest amount of injury occurred with cis-2-butene followed by propylene, 1-hexene, and cumene. Of particular significance is the fact that for the first time 2-carbon products are responsible for oxidant damage. This was not so with the original studies of Haagen-Smit and co-workers and the somewhat later studies of Darley and associates in which they dealt solely with ozone-olefin dark reactions and were unable to produce oxidant injury by oxidizing 2-butene and propylene. Stephens *et al.* (47) also demonstrated that phytotoxic materials were produced from the irradiation of aldehydes of the species resulting from the products of ozone-olefin reactions. These investigators further showed that in addition to the photooxidation of aldehydes and the production of plant injury, peroxy acids resulting from this photooxidation were not able to produce typical oxidant damage. They do, however, confirm earlier findings of Haagen-Smit *et al.* (21) that hydrogen peroxide in very high concentrations may cause typical oxidant injury. It is interesting, indeed, that the oxidant injury incited by hydrogen peroxide occurs only on the 14-day-old primary leaves of bean and not on the primary leaves of 8-day-old beans suggesting that hydrogen peroxide is more closely related to the phytotoxicant resulting from ozone-olefin dark reaction products than it is to that from the nitrogen dioxide-olefin light reaction products.

Ozone

Hydrocarbon alone does not cause plant damage. Ozone is produced from the photolysis of hydrocarbon in the presence of nitrogen dioxide. The ozone from this reaction causes plant damage. Middleton *et al.* (38) report injury from nitrogen dioxide only when concentrations were above 3 p.p.m. for about 4 hr.

Symptoms of ozone injury.—Middleton *et al.* (40) summarized reports of ozone injury to vegetation in 1955 and showed that Knight & Priestley in 1914 were able to incite injury to brussel spouts, pea, rye, and wheat. The bleaching of bean leaves by ozone was reported about 20 years later by Homan. Middleton (35) reported that the upper leaf surface of bean leaves became bleached in the presence of ozone under controlled fumigations, thus corroborating the earlier reports of Homan. The environment under which the plants were produced determined whether the leaves became bleached or developed reddish-brown to dark brown punctate spots, all symptoms being limited to the upper leaf surface. A linear relationship between concentration and time of exposure was determined for ozone and its damaging effect upon sensitive vegetation. Although ozone injury to experimental plants under controlled conditions can be demonstrated easily and the injury is severe at 0.5 p.p.m. of ozone for a half-hour, ozone was of no economic importance to field grown plants before 1954 but began becoming serious with agricultural plants in 1956 in California. More recently Richards *et al.* (46) demonstrated that the upper leaf surface dark brown to black punctate spotting of grapes

was attributable to the presence of ozone in vineyards. This disease has since become of considerable economic importance in southern California and is becoming important in other areas where photochemical air pollution occurs in California. Heggestad & Middleton (22) reported that a chlorosis and an upper leaf surface flecking of tobacco, formerly known as "weather fleck," was induced by ozone. This disease has been observed in the eastern seaboard of the United States and presents a serious problem in the production of cigar-wrapper tobacco in the Connecticut valley and other tobacco-growing areas farther south. These authors report that tobacco varieties vary in their resistance to the disease in the field and that these differences are corroborated by experimental fumigation and exposure to ozone under laboratory conditions. The occurrence of ozone injury to a variety of vegetables, especially spinach, was reported in New Jersey by Daines *et al.* (10, 11). In each of these instances the upper leaf surface was injured and was typically chlorotic. The tissues of affected leaves very often subsequently became necrotic and sunken. Hill *et al.* (23) reported the effect of ozone upon 34 plant species under controlled greenhouse atmospheres. They indicated that the most sensitive species were the small grains, alfalfa, spinach, and tobacco, and that a general tendency existed for sensitivity to increase with maturity of tissue. They point out, as with oxidant, that injury can occur on either leaf surface of plants that have undifferentiated mesophyll. In the instance of broccoli they reported that ozone caused conspicuous tumors that were especially pronounced on the lower leaf surface.

Ledbetter *et al.* (29) have shown a number of plants to be sensitive to ozone, have described the symptomatology, and have corroborated earlier findings that ozone injury is typically confined to the upper leaf surface and notably to the uppermost layer of palisade cells and epidermis. They reported that very young leaves without functional stomates were resistant to ozone whereas those leaves with functional stomates were susceptible; this response is identical with that to oxidant. Taylor *et al.* (51) corroborated some of these findings by demonstrating that severe injury from ozone occurred on the oldest leaves near the base of the plants with progressively less injury to young foliage. The amount of injury to the foliage was not only related to the age of the leaf but to the concentration and the length of exposure time.

As with oxidant, environmental factors modify both occurrence and intensity of ozone effects upon sensitive vegetation. Taylor *et al.* (48) showed that weather fleck of tobacco caused considerable damage whenever combinations of heavy rainfall, irrigation, slow drainage, or low transpiration led to a low soil moisture stress; they were unable to correlate injury with stomatal aperture.

Physiological effects of ozone.—Todd & Garber (57) have shown that fumigation of Alaska pea with ozone had no effect upon plant height and that plant growth was not suppressed by exposure to ozone. Erickson & Wedding (17) show that duckweed exposed to ozone had a photosynthetic rate of about 60 per cent of the controls, and further, that ozone did not stimulate respira-

tion. They remarked that the reduction in chlorophyll content of the exposed plants could account for the reduced rate of photosynthesis. The increased conductivity of the water in which the plants were floated during exposure to ozone showed that after a 24-hour exposure about 25 per cent of the cells lost their semipermeability.

Todd (56) has shown that bean leaves fumigated with ozone without the development of visible injury showed no increase in respiration and no inhibition of photosynthesis. However, when the bean leaves were damaged by exposure to ozone, both the amounts of stimulation of respiration and the inhibition of photosynthesis were directly related to the development of the injury symptoms. This was not true with leaves of Valencia orange since the respiration rate increased with no concomitant development of visible injury. Todd suggests that the citrus leaves may have had cellular injury which was not macroscopically detectable, and that the citrus leaf was capable of promptly repairing this injury. These observations on citrus leaves parallel the response Todd (54) obtained in experiments with lemon fruit where a respiratory stimulation elicited by ozone was not accompanied by any obvious fruit injury. Todd (55), in a further attempt to elaborate upon the mechanism of action of ozone upon photosynthesis and respiration, studied the action of ozone on the enzymes catalase, peroxidase, papain, and urease. He found wide variations in the sensitivity of the enzymes in the *in vitro* tests to low concentrations of ozone, but papain was most sensitive, with the others requiring a much greater amount of ozone for activation. A comparison of ozone and hydrogen peroxide as inhibitors of papain and urease showed that ozone was 30 times more effective than hydrogen peroxide on papain and 3 times as effective on urease.

Perhaps the most interesting recent development on the biochemical effects of ozone upon cellular activity is the report of Freebairn (19). He used mitochondrial preparations from cabbage and spinach as well as from cow's liver. To some samples he added reducing compounds and then passed through the preparations a stream of ozone or air in *in vitro* tests. The results suggest that the effects of small amounts of ozone on the mitochondrial preparations were reversed by ascorbic acid, as well as reduced glutathione. From this work he proposed that the ozone-affected enzymes were associated with the mitochondrial citric acid cycle activity and that some specific toxicity system was operating.

Occurrence and Distribution of Ozone and Oxidant Plant Damage

The typical and unique lower leaf surface injury to dicotyledon leaves attributable to ozone-olefin dark and light reaction products makes it possible to use plants to detect the presence of these phytotoxicants wherever susceptible vegetation is grown. Likewise, the unique and typical upper leaf surface spotting and chlorosis due to the effects of ozone make it possible to use sensitive plants as indicators for the presence of this air-borne phytotoxicant resulting from photochemical reactions. Although Bobrov (6) used

annual bluegrass (*Poa annua*) as particularly sensitive to community-type air pollutants, it cannot be used as an indicator for the separation of these two phytotoxicants, as both oxidant and ozone produce identical leaf symptoms on this host. The original report by Middleton *et al.* (42) showed that this oxidant injury first occurred in the southwestern Los Angeles area and over a period of six years occupied most of the south coastal plain in the vicinity of Los Angeles. Five years later Middleton (34) reported that the entire south coastal basin of southern California was plagued by phytotoxic levels of oxidant. Went (60) reported in 1955 that he had seen the effects of oxidant (smog) on vegetation in the following cities: Bogota, Colombia; Cologne, Germany; Copenhagen, Denmark; London and Manchester, England; Paris, France; and Sao Paulo, Brazil. He additionally reported the presence of these phytotoxicants in Baltimore, Maryland; Los Angeles, California; New York City, New York; Philadelphia, Pennsylvania; and San Francisco, California.

The usefulness of plants in reporting the presence of pollutants was used by Middleton & Paulus (43) in conducting a survey of plant damage from air pollution throughout California, utilizing a mechanized system of assembling reports made manually in the field. They presented both black-and-white and colored illustrations of injury; this allowed the separation of damage due to ethylene, fluorine, oxidant, ozone, and sulfur dioxide. The toxicants responsible for plant damage as a result of this survey were recognized in decreasing order of importance as oxidant, ethylene, and fluorides. Utilizing the elements of this program, Middleton (36) gave more recent information showing that within California the reaction products of ozone-olefin and photochemical oxidation of hydrocarbons are now present in 27 counties in the state ranging from the south coastal basin of Los Angeles and San Diego to the San Francisco bay area, the San Joaquin and Sacramento delta, and lower portions of the central valley. Community-type air pollution now affects 11,000 square miles in the state; 44 per cent of this occurs in central California, 40 per cent in southern California, and 16 per cent in the delta and San Joaquin valley area.

The most recent account of the distribution of photochemical air pollution is that of Middleton & Haagen-Smit (39) in which they reported the toxicants present in 19 states (Arizona, California, Colorado, Connecticut, Delaware, Hawaii, Illinois, Indiana, Maryland, Massachusetts, Missouri, North Carolina, New Jersey, New York, Ohio, Pennsylvania, Utah, Washington, and West Virginia), District of Columbia, and the Province of Ontario, Canada, as well as in the northern portions of Baja California, Mexico. They pointed out the growing importance of photochemical air pollution in the eastern seaboard of the United States, especially in the Connecticut and Delaware River valleys, and indicated that ozone appears to be a more important plant damage factor than oxidant on the eastern seaboard, while the reverse is true on the western seaboard.

The economic significance of plant damage from these toxicants has not

been thoroughly studied. Considered estimates of visible damage to agricultural crops amount annually to about $8,000,000 in California for field and vegetable crops and approximately $18,000,000 along the Atlantic seaboard. No estimates have yet been made of the real economic loss due to growth suppression, delayed maturity, and the attendant increased cost of crop production and reduced yields.

Conclusions

Air as a natural resource becomes contaminated depending upon weather, land contour, air drainage, and the kind and amount of emissions from community activities. Injury to vegetation is usually of considerable economic significance, both to food, flowers, and ornamentals in the polluted communities, and to the agricultural crops in the contiguous rural areas plagued by photochemical air pollution arising from urban areas and associated communities. The symptom development resulting from plant injury by ethylene, fluorine, and sulfur dioxide shows that the mechanism of action of these contaminants is different than that caused by ozone and oxidant. The ozone and oxidant resulting from the photooxidation of nitrogen dioxide and hydrocarbons injure plants, whereas the nitrogen dioxide and hydrocarbons participating in this reaction are typically noninjurious. Chemical methods for measurement of ozone and oxidant usually do not allow discrimination between these two classes of toxicants. Middleton *et al.* (40) showed that leaves of dicotyledons do discriminate between these toxicants, for ozone affects the upper leaf surface and oxidants the lower; these types of damage are often observed on the same leaf. The highly reactive ozone molecule causes damage to the upper leaf surface by destruction of the upper palisade cells and their chloroplasts. This injury is all the more interesting since it occurs in the complete absence of stomates on the upper leaf surface requiring that the ozone in cutinized leaves enter solely through the lower leaf surface and traverse the entire, wetted, intercellular spaces of the mesophyll prior to affecting the areas of the leaf opposite the point of toxicant entry.

Heretofore most workers have considered that the ozone-olefin dark reaction products were similar to the products of the nitrogen-dioxide—hydrocarbon light reaction. The report of Taylor *et al.* (51) clearly demonstrates that the oxidants resulting from these two separate reactions, although capable of affecting a given species of plant, nonetheless affect different ages of leaves. It follows that despite the similarity in symptoms there are at least two chemical compounds responsible for this injury. This suggests that oxidant symptom development in plants is inadequate for the separation of chemically different phytotoxic, oxidant-type pollutants. This view is also supported by the report that the oxygenated hydrocarbon molecule of acrolein and the simple hydrogen peroxide molecule produce injuries at relatively high concentrations that are indistinguishable from those incited by some other oxygenated compounds. Stephens *et al.* (47) report one of these phytotoxicants to be peroxyacetyl nitrite, whereas Darley *et al.* (13)

were unable to specifically identify the phytotoxicant from ozone-olefin dark reactions and suggest that it may be a zwitter ion or some other unstable species. Oxidized hydrocarbon contaminants in community air therefore contain at least three materials capable of releasing iodine from potassium iodide and causing plant damage, namely: ozone, peroxyacetyl nitrite, and an unidentified reaction product.

These different toxicants in community-polluted air also have different physiological effects upon plants in addition to different symptom expressions. A comparison of the effects Todd & Garber (57) observed with ozone and ozonated hexene upon pea shows that ozone has no growth suppressing effect after exposure whereas ozonated hexene has a strong suppressive effect on growth. Erickson & Wedding (17), and Todd (56) showed that photosynthesis was not inhibited in either duckweed or bean when the plants were exposed to ozone in the absence of visible injury to tissue. It is also apparent that there was no increase in respiration from ozonated hexene or ozone without visible injury. The work of Todd related to the expansion of bean leaves suggests that growth suppression is related to inhibition of cell expansion. Perhaps the lack of elongation and expansion is due to the decreased permeability of cells to water, as shown by Wedding & Erickson (59). Although there is as yet insufficient work to attempt to relate the inhibitory effect of ozone upon oxygen uptake by mitochondria and its influence upon respiration and growth of plants, it seems likely that a specific toxicity exists. This, too, may inhibit normal cell elongation without affecting cell numbers.

Other modifying influences affect the development of ozone and oxidant symptom expression. These have been earlier reported as both pre- and post-temperature effects, soil moisture, fertility level, and light. There are, however, two other interesting modifying factors that affect oxidant and ozone damage to plants. Kendrick *et al.* (26, 27) showed that typical oxidant damage to beans could be prevented by topical applications of sprays or dusts of carbamates and related compounds (such as Zineb, Ferbam, Ziram, Maneb, and Thiram) to plants. The amount of damage to beans treated with these materials increased with an increase in time between application and exposure to the phytotoxicant. It was also demonstrated by Kendrick and associates that the injury increased progressively on the protected plants with increased toxicant-exposure times. Further, the protection phenomenon was evident only when the leaf surface presenting stomates was adequately covered by the effective chemical. Rich & Taylor (45) have shown that antiozonants are able to protect tomato foliage from damage from ozone when the material is placed on cloth and the test cloths placed about the plant. In the case of the tomato and the bean, both exemplify a preventing of the phytotoxicant from reaching plant tissue. Freebairn & Taylor (20) report that plant damage by the oxidants can be prevented by ascorbic acid through the application of potassium ascorbate as a liquid drench to sensitive indicator plants. The effectiveness of the ascorbate is a function of the amount of material taken up by the plant, the dosage of oxidant and ozone to which the

plant is exposed, and the length of time between application and first exposure to the plant-damaging toxicant.

Still another interesting protection phenomenon is that reported by Yarwood & Middleton (61) in which bean rust prevented oxidant injury about the rust postules on bean leaves. The same level of protection was provided the infected bean leaf even after the rust fungus had been killed in the leaf by hot water treatment insufficient to affect host cells. Since it was known that bean rust greatly increased the pantothenic acid content of bean leaves, attempts were made to protect bean leaves from oxidant injury by the application of pantothenic acid. All efforts to protect bean foliage from oxidant damage by pantothenic acid were unsuccessful; the nature of rust protection remains unknown.

Control of photochemical air pollution.—The ever mounting damage to agricultural crops and vegetation throughout the United States, the steadily increasing number of new communities suffering from photochemical air pollution, and the growing concern over the effect of this kind of pollution upon the public health and well-being have been responsible for the initiation of air pollution control programs. Although the level of government responsible for air pollution abatement differs with states and community interests, stationary sources of pollution are usually controlled by municipal or county agencies. Middleton (36) has recently described the evolution of air pollution abatement in California and the newly assumed role of the State of California in the control of hydrocarbon emissions from motor vehicles, which are believed to be the principal source of most of California's photochemical air pollution. This significant step towards control of hydrocarbon sources was made possible because automobile exhaust had been indicted as a source of pollution by many investigators including Faith (18), Middleton & Haagen-Smit (39), and Stephens *et al.* (47), and because first standards for acceptable vehicle exhaust emissions as well as ambient air were established for California by Merrill (33). The establishment of these standards is an essential first step toward the control of photochemical air pollution and can be expected to effect an improvement in the quality of air. It is, however, only a first step in a forward direction, for although reduction in hydrocarbon emissions will in turn undoubtedly reduce levels of oxidant and, in turn, oxidant damage to vegetation, it is unlikely that ozone levels will be sufficiently reduced. Residual ozone may become the important plant damaging factor in California if hydrocarbons are reduced to levels that eliminate their effectiveness as atmospheric scavengers for the ozone formed from the photolysis of nitrogen dioxide in sunlight. Middleton & Haagen-Smit (39) have postulated that the change in manifestations of photochemical air pollution from one involving oxidant effects in the urban area, to oxidant and ozone in the surburban areas, and to ozone in rural areas, is due to changes in the chemical composition of the moving pollution cloud as associated with reaction time and coupled with the relatively prompt consumption of olefins, slower consumption of a variety of organics, and delayed consumption of

saturated hydrocarbons, so that ozone production continues from the photolysis of nitrogen dioxide some distance away from the initial, principal pollution source. The manifestations of photochemical air pollution in and about Washington, D. C., and Philadelphia, Pennsylvania, conform with the thesis postulated here. Since the oxides of nitrogen arise from any hot combustion of fuel in air and are primary ingredients in the photochemical air pollution system, ultimate success in abatement of community air pollution requires control of the nitrogen oxides emitted into the atmosphere. The present air pollution abatement program outlook in California suggests a reduction in plant damage from oxidants with a possible increase in plant damage from ozone unless steps are taken to control nitrogen oxides. For, while hydrocarbon controls will effectively reduce atmospheric hydrocarbon levels, it is likely that sufficient residual hydrocarbon would still remain to then possibly participate in the cyclic evolution of ozone through photo oxidation of nitrogen dioxide; concurrent control of nitrogen oxides would effect, therefore, a reduction in atmospheric ozone levels.

The development of standards for ambient air quality are essential for the conservation of air as a limited natural resource and the protection of our agricultural economy through the prevention of photochemical air pollution damage to plants.

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PHYSIOLOGY OF WILT DISEASE[1,2]

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Despite the many approaches that have been made to the problem of the "cause and effect" of wilt pathogenesis, we have a long way to go before we understand the many complex situations in the sequence of events that culminate in the syndrome. Many thoughtful reviews have been written by Brian (14), Dimond (27), and Gäumann (36, 37) on the multi-faceted nature of this problem and although some new aspects are presented here, it seems essential also to cover some of the well-known land marks to permit a proper appreciation of the numerous pitfalls that confront a wilt disease worker. While many *in vivo* changes in the host subsequent to root infection have been observed, the interpretation of these derangements in metabolism has been rendered difficult except in one or two cases where the actual role of the products of fungal metabolism is known. However, with increasing awareness of the need for developing new techniques for bridging this gap in our knowledge, we can now look forward to deeper penetration into the biogenesis of toxin formation (in the rhizosphere and *in vivo*) and its effect on the functional norm in host tissue.

WILT TOXINS AND VIVOTOXINS

Among the wilt toxins currently being studied, fusaric acid occupies an important place (36, 37). This is because it is now recognised as a vivotoxin [Lakshminarayanan & Subramanian (81)]. Much space is devoted to a consideration of this vivotoxin, as our knowledge of it is comparatively complete, although other toxins like alternaric acid and lycomarasmin also show interesting features from the view point of toxin production and uptake by plants (14).

Fusarium vasinfectum Atk., the cotton wilt pathogen, produces fusaric acid (5-*n*-butylpyridine carboxylic acid with the formula $C_{10}H_{13}O_2N$ and a molecular weight of 179) in sterilised soil with organic amendments. Fusaric acid, a host-aspecific wilt toxin, is also produced by *Fusarium oxysporum* f. *lycopersici*, *F. heterosporum*, *Gibberella fujikuroi*, and *Nectria cinnabarina*. Quite recently, *F. oxysporum* f. *cubense* was shown to produce it *in vitro* and it was also detected as a systemic vivotoxin in rhizomes of banana plants infected by this fungus (95). The maximum quantity of fusaric acid detected in soils is in the order of 7.9 μg., expressed as fusaric acid equivalent, per gm. of soil [Kalyanasundaram (60)]. A point of further interest about this wilt toxin is that it has been detected in the rhizosphere of tomato plants growing in

[1] The survey of literature pertaining to this review was completed May 1960.

[2] The following abbreviations are used: DP (depolymerase); IAA (indole-3-acetic acid); IAAe (indole-3-acetic acid equivalents); NPN (non-protein nitrogen); PG (polygalacturonase); PME (pectin methylesterase); PN (protein nitrogen).

unsterilised acid-washed sand and soil inoculated with *F. oxysporum* f. *lycopersici*. Approximately 7.5 μg. and 11.25 μg., respectively, of the toxin were present in the rhizosphere of 20-day-old plants. Detection was possible even 48 hr. after inoculation, by which time the pathogen hardly made any growth internally in the host. Also, about 20 per cent of the original amount of fusaric acid could be detected after 10 days' incubation in soil, indicating its persistence [Kalyanasundaram (61)]. It is obvious that root exudates offer enough substrate for the fungus to grow and synthesise its products of metabolism. In fact, analysis of tomato root exudates showed the presence of many amino acids, glucose, and certain other reducing substances. As this fungus has also been shown to be capable of using amino acids (2-^{14}C-glycine) almost as the sole source of energy for growth and synthesis of toxins [Sanwal (104)], it seems gratuitous to postulate possibilities of alternate sources of energy when the existing data on energy substrates available in the rhizosphere would seem to indicate that the basic metabolic needs of the pathogen are met. Thus, many factors in the rhizosphere play a significant role in the wilt syndrome; these will be discussed later.

Quite recently the new concept of a vivotoxin has developed. A vivotoxin is defined as a substance that is produced in the infected host by the pathogen or its host or by both, and that functions in the production of disease, but is not itself the initial inciting agent of disease [Dimond & Waggoner (28)].

The first typical wilt toxin is the much quoted ethylene, known to be produced by injured plants [Hall (45, 46)]. Ethylene itself injures cells and stimulates self-propagation, functioning as an autocatalyst. Ethylene originates in ripening fruits from the degradation of intracellular substrates like simple sugars or organic acids and this leads to the hydrolysis of complex insoluble cell-wall constituents (such as pectin compounds and hemicelluloses) to soluble ethylene-precursor substrates which in turn are degraded during respiration. Pectin, therefore, emerges as an ethylene-precursor substrate. Another good substrate for ethylene appears to be ethanol. Thus, "potential" ethylene-yielding substrates are eventually reduced to alcohol which in turn appears to be the terminal substrate for ethylene production. It should be stated, however, that pectin hydrolysis and ethylene production are not correlatable [Ludwig (85)]. The main function of ethylene is considered to be that of indirectly uncoupling respiration from energy-requiring cell activities. Despite this last observation, the role of pectin, now well recognised in wilt pathogenesis, may have to be more critically studied in relation to vivotoxins and ethylene production since a competition for the same substrate(s) may eventually be found. Further, increased auxin reported under pathogenesis in *Verticillium* wilt of tomato may have to be reviewed in light of these observations on ethylene production. *Verticillium* toxin also may eventually turn out to be a vivotoxin since its disease syndrome has much similarity to fusariose diseases.

The result of wounding in the roots is also known to produce *in vivo*

wound hormones, particularly traumatic acid (a dicarboxylic acid) and possibly IAA (indole-3-acetic acid). Other dicarboxylic acids that are active in varying degrees as wound hormones have also been synthesised [Bloch (11)]. It would undoubtedly be of great interest if we could understand the formation of these dicarboxylic acids *in vivo* in plants suffering from pathogenic wilt syndrome. Traumatic acid, glutathione, and other growth substances were tested for wound healing activity in *Acer saccharum* and of these, only glutathione was found to stimulate wound healing [Davis (24)]. The pathologist could probably now look forward to studying glutathione and related substances *in vivo* in wilting plants as specific sequelae of wilt syndrome.

Increases of up to 200 per cent in growth-promoting activity assayed as IAAe (indole-3-acetic acid equivalents) in stems and leaves of tomato plants infected by *Verticillium albo-atrum* over healthy controls have been observed [Pegg & Selman (98)]. *V. albo-atrum* cultures showed growth promoting activity when assayed and, indeed, many fungi are known to synthesise auxins in culture (44). Disease symptoms induced by *V. albo-atrum* partly simulate those caused by the ethylene syndrome and as it is well known that, for a given response the molecular ratio of ethylene to IAA may be constant (11), critical estimation *in vivo* of these two substances may stimulate new thinking. This ratio may have to be determined not only in *Verticillium* wilt but also in other wilts where increased IAAe *in vivo*, consequent to infection, has been recorded.

Unlike ethylene, which increases in quantity *in vivo* with greater wounding, the production of fusaric acid seems to depend on factors operative in the rhizosphere and on the metabolic state of the host. In fact, the sensitivity of *Bacillus subtilis* and *Escherichia coli* to fusaric acid in organic media was less than 10 per cent of that in a synthetic substrate and this lowered potency is ascribed to an antagonist in the organic media [Kalyanasundaram (62)]. Further, this antagonist was capable of removing the toxicity of fusaric acid to tomato shoots. It appeared that the minimum dose of fusaric acid needed to cause injury to any organism depended on the proportional distribution of the antagonist and this probably explains the differing sensitivity of various host plants to fusaric acid. Thus, it seems that a lacuna exists in our knowledge of *in vivo* production of ethylene, traumatic acid, IAA, and other possible intracellular toxins, and antagonists to vivotoxins resulting from wounding of the root by wilt fungi or other agents that cause damage to root tissues.

FACTORS GOVERNING ROOT INFECTION AND ESTABLISHMENT

During the invasion of roots whereby a vascular wilt pathogen establishes infection, an opportunity exists for soil conditions to exert a direct effect upon the activity of a parasite; however, their effect via changes in the host physiology is greater by comparison [Garrett (34)]. In general, wilt diseases are

favored by high temperatures (25–30°C.) and by acid soil conditions with a high nitrogen and a low potassium supply. In addition, other factors such as the occurrence of nematodes also increase the incidence of wilt diseases. The effects of soil microflora, especially in the rhizosphere of host plants, represent an important biotic factor.

Nematodes and wilt incidence.—Atkinson (5) was one of the earliest to suggest a relationship between root-knot nematodes and *Fusarium* wilt of cotton. Strains bred for wilt resistance invariably fail when the soil is infested with nematodes and wilt pathogen (47, 90, 116). The complicity of nematodes in increasing wilt incidence appears to be by providing avenues of entry into the host roots [Lucas *et al.* (84); Newhall (91)], or by causing certain specific responses in roots that predispose them to infection [Martin *et al.* (87); Jenkins & Coursen (54)]. The association of nematodes with plant roots is rather specific and seems to be promoted by dialysable root exudations [Lownsbery & Viglierchio (83]. Nematodes, therefore, constitute an important biotic factor. Much work lies ahead in this direction and critical study of responses incited by nematodes on host roots and their effects on wilt pathogens, all living in a dynamic environment, will be amply rewarded.

Rhizosphere.—The "rhizosphere" is now recognised as a special ecologic habitat subject to the specific influence of plant roots. This new concept of root exudations affecting microfloras of the rhizosphere quantitatively and qualitatively has helped much in the study of soil-borne root diseases. It is not difficult to envisage the impact of these concepts on wilt disease investigations. The rhizosphere microflora exerts a dynamic influence on root-infecting pathogens and this in turn is influenced by the host. The conditions in the rhizosphere may be such that the survival, growth, and production of enzymes and toxins by the pathogen become impossible due to the presence of toxic substances, inhibitors, or antagonistic microorganisms. Flax varieties resistant to wilt produce sizeable amounts of hydrocyanic acid toxic to *Fusarium oxysporum* f. *lini* while the suscepts exude very little of this inhibitor. In the rhizosphere of a resistant variety, *Trichoderma viride*, a well known antagonist, thrives to the exclusion of other forms that are sensitive to HCN [Timonin (133)]. Agnihothrudu (2) working on pigeon-pea wilt showed that the causal organism, *Fusarium udum*, was not favored by the resistant variety in the rhizosphere due to a predominance of antagonistic actinomycetes. The chances of survival of *F. moniliforme*, which causes foot-rot in rice (*Oryza sativa* L.), is twentyfold less in the rhizosphere of a resistant variety than in that of the suscepts [Bhuvaneswari (10)]. Buxton's work on this aspect is stimulating. Root exudates of pea variety Alaska, resistant to race 1 of *Fusarium oxysporum* f. *pisi* inhibited spores of race 1 but not those of race 2 which is pathogenic on that variety; on the contrary, spores of race 2 were stimulated. However, if the spores of race 1 are retained in the exudates from Alaska pea roots for 14 days they become as pathogenic as race 2 toward that variety; this effect is greater if the concentration of the exudate is increased

fourfold. Mutation or enzymatic adaptation are two possibilities for this change in the pathogenic behaviour of race 1, but since the treatment does not yield a mixture of races 1 and 2, a mutagenic effect of the exudate is excluded [Buxton (19)].

Much of the work on rhizosphere microfloras and root exudates from host plants resistant or susceptible to wilt diseases indicates that the susceptible plants exert a more marked rhizosphere effect and exude higher quantities of amino acids, sugars, vitamins, etc., not to mention the qualitative differences (2, 10, 17, 18, 126). It is clear that the rhizosphere region of the susceptible host varieties, in most cases so far investigated, is richer and more conducive to the growth of microorganisms in general and wilt pathogens in particular than that of resistant varieties. The question is whether this nutritionally rich and varied micro-habitat, the rhizosphere, plays a more determinate role in the development of root-diseases than is understood at present. Does it form a springboard from which the root-infecting wilt pathogens build up their potential for attack? Do the exudations provide any chemotropic stimulus to prospective pathogens? Is the attainment of an ecologic "dominance" by a pathogen in the rhizosphere of its host a prerequisite to invasion? Does the nature of root exudates exert any selective action on the species or race that ultimately infects that host? Or, alternatively, is host specificity a character imposed on the root-infecting pathogen in the rhizosphere by the nature of root excretions? Environment does seem to exert a far reaching influence on microorganisms by evoking variations and adaptations in them, whatever be the underlying mechanisms. In wilt fungi particularly, despite the absence of a regular occurrence of a sexual stage, a great deal of pathogenic race differentiation goes on through heterokaryosis, parasexual recombination, and saltation, not to mention the phenomena of enzymatic adaptation and mutation [Buxton (15, 16)]. It would be a very fruitful undertaking to study the pathogenic physiology of root-infecting fungi as affected by substrates available in the rhizosphere. That the tomato wilt *Fusarium* could grow in the rhizosphere and root surface of susceptible tomato plants and produce detectable quantities of fusaric acid (61), and that it could use amino acids alone as a source of both carbon and nitrogen (104) are of particular interest. Since many soil microorganisms produce fairly stable antibiotics and toxins *in situ* which are taken up by plants, it would seem that the chain of events in wilt pathogenesis starts from the moment the pathogen gains ground in the rhizopshere of the susceptible host, that is, prior to infection itself.

Root infection.—Vascular wilt pathogens enter their hosts through wounds on the stem and in the root-infecting types through young roots and rootlets. More often they find other avenues of entry such as wounds in the root cortex caused by emerging rootlets, nematodes, etc. Subsequent spread and generalisation seem to depend on the number of roots that are infected; the more their number, the greater are the chances of overcoming resistance

offered by tissue reactions [Rishbeth (100)]. Effective root infection appears to play a very determinate part in directing the course of later phases as indicated by grafting experiments. Roots of a low-resistance tomato variety contained more infected vessels than the high-resistance variety when infected with *Fusarium oxysporum* f. *lycopersici* (117). That resistance or susceptibility is determined by the root stock is indicated both in tomato [Heinze & Andrus (48)] and in hop [Keyworth (67)].

The important role of pectolytic enzymes in wilt disease has been emphasised by many (52, 64, 79, 135). While some wilt pathogens secrete these enzymes constitutively, certain others do so only adaptively. Work on *Fusarium* wilt of cotton has shown some interesting correlations. Secretion of these enzymes has been noticed to be partly adaptive in nature (78). The pectin reserves, especially in roots of wilt-susceptible *Gossypium arboreum* var. *indicum*, are higher than those available in resistant *G. hirsutum* strains. It is postulated that the nonavailability or availability in sub-optimal concentrations of substrate governs the production of these enzymes *in vivo*. Work in this laboratory also revealed that secretion of pectolytic enzymes by *Fusarium vasinfectum* is controlled by sugar concentrations in pectin media; a high concentration tends to inhibit while a low level augments [Subramanian (123)]. Cellulase secretion by *Verticillium albo-atrum* is influenced by sugar in a similar manner (129). It could, therefore, be postulated that the availability of critical concentrations of effective carbon (sugars) may govern the establishment of pathogens *in vivo*.

Polyphenol oxidases have often been implicated in resistance to plant disease and Cole (21) showed that the products resulting from the action of these enzymes are inhibitory to pectolytic enzyme activity. Fusaric acid, a vivotoxin in tomato and cotton wilts, has recently been shown to competitively inhibit polyphenol oxidase [Bossi (13)]. It is likely that such an action of fusaric acid aids the uninhibited action of pectolytic enzymes in fusariose wilts of tomato and cotton.

The effect of nitrogen in promoting vascular wilt diseases seems to be almost general (34). The beneficial effects of potash amendments of soil in controlling wilts of plants were explained by their stimulating the synthesis of organic derivatives of nitrogen and the consequent diminution of inorganic nitrogen in the trachea [Shear & Wingard (114)]. A variety of cotton, susceptible to *Fusarium* wilt, accumulated NPN (non-protein nitrogen) in roots while the resistant variety accumulated PN (protein nitrogen). NPN appears to be a most favored nitrogen substrate for the cotton wilt organism *in vitro* [Satyanarayana (107)]. Therefore, a plentiful supply of NPN in roots appears to be ideal for the establishment of the pathogen in cotton wilt. Recent work of Selman & Buckley (111) on factors affecting *Verticillium* invasion of tomato roots is highly corroborative. A low nitrogen supply, which leads to an increase in internal sugar concentration, limits the rate of

systemic invasion whereas an increased nitrogen supply apparently has no effect. Although these authors contend that the effects of low nitrogen on the rate of systemic invasion result from its influence on the host physiology, we believe that it is the result of high internal sugar concentration affecting enzyme secretion by the pathogen.

THE DISEASE SYNDROME

The remarkable similarity in disease syndrome in the various wilt diseases, whether caused by pathogens as varied as fungi, bacteria, or viruses, certainly suggests some common biochemical basis. Most of the pathogens tend to be confined to the vascular elements in early stages and invasion or disintegration of other tissues are not evident until later.

External Symptoms

The generally reported symptoms are: dwarfing and stunting, leaf yellowing, and necrosis—starting either as vein-clearing, mottling or general yellowing; epinasty, defoliation, root formation from the stem, and loss of turgor—with the wilting foliage often recovering at night in the early stages before permanently wilting; collapse and death of the diseased plants. The syndrome will, indeed, vary considerably and depart in many ways from this general sequence. The disease may progress slowly, or there may be a sudden wilting; in some cases one or more of these symptoms may be lacking. These may develop multilaterally involving the entire plant, or unilaterally affecting only a portion of the shoot. There does not seem to be a perfect correlation between the site of entry of the pathogen and the severity and sequence of symptom development. Some soil-borne root-invaders cause a typical unilateral wilt (*Fusarium*), while branch invaders sometimes destroy entire plants (wilt in red oaks). Again, in the majority, the symptoms are noted to progress from below upwards, but there are exceptions such as the bacterial wilt of bananas caused by the soil-borne *Pseudomonas solanacearum*, where the inner leaves are the first to wilt [Sequeira (113)]. The bacteria themselves were found to be concentrated towards the rhizome apex. The varied symptom expression is evidence in itself for the pathogens and their metabolic products playing an important role in wilt pathogenesis.

Fluorescence and vein clearing, the very early symptoms.—These have been reported only in very few cases. Clearing of the ultimate veinlets giving the leaflets a "netted" appearance, visible only in transmitted light even 24 hr. after inoculation, has been reported for *Fusarium* wilt of tomato (33). Vein clearing has been noted in the *Fusarium* wilt of cotton and the *Verticillium* wilt of garden stock [Kalyanasundaram (58); Raade & Wilhem (99)]. The cleared leaf veins in cotton fluoresced bright yellow in ultraviolet light. This fluorescence was noticed 5 to 6 days earlier in hypocotyl, stem, petiole, and vein sections and was found mainly in the vascular regions [Subba-Rao

(120)]. Fluorescence of lesions in early stages is also known to occur in the virus diseases, tomato spotted wilt and potato leaf roll. The fluorescing substance has been isolated in crystalline form and named scopoletin [Andreae (4); Best (7, 8)]. Since the fungal pathogens were not present in symptomatic portions, it is evident that fungal metabolites are active. This conclusion is further strengthened by the detection of radioactive substances in inoculated host tissues far removed from the ^{14}C-tagged pathogen used [Kern & Sanwal (66)]. Although the nature of the substance in cotton wilt is not definitely known, it is presumed to be some product of host tissue degradation [Subba-Rao (121)]. Phenols, liberated into vessels of wilting plants [Davis *et al*, (25)], are known to have fluorescing properties.

Epinasty, yellowing, defoliation, and adventitious root formation—Ethylene, already considered a vivotoxin (27), is known to produce all the above-mentioned symptoms in plants. The possibility, however, of other growth substances also being involved needs consideration. For instance, some of the foliar symptoms of oak wilt (including downward bending of leaves and premature leaf fall) simulate the ethylene effect, but *in vitro* the pathogen revealed no ethylene production; instead, a growth substance was noticed [Fergus & Wharton (32)]. Many of the symptoms of *Verticillium* wilt of tomato could be induced in shoots by supplying IAA, and considerable quantities of IAAe were detected in inoculated plants (98). Since the autocatalyst, ethylene, is known to be produced by tissues of higher plants under the slightest irritation, it is possible that ethylene is involved in epinasty, yellowing, etc. An auxin-ethylene balance, as suggested by Hall (46), would seem important in defoliation, as not in all cases do the symptomatic leaves drop off, e.g., pigeon-pea wilt. Whether mere shortage of water inside the stem would induce yellowing and epinasty, as reported by Threlfall (132), needs confirmation.

Dwarfing, stunting, and necrosis.—In many instances the infected plants are seen to be much stunted, with reduced root systems, shorter internodes, and a consequent crowding of small dwarfed leaves (30). Reduction in the leaf area was evident rather than a fall in the rate of leaf production, and this was thought to be an effect of toxins (112). Toxic metabolites of the pathogen were again implicated in the desiccation and necrosis in the *Verticillium* wilt of hops [Talboys (127, 130)].

The severity and expression of these symptoms are quite variable according to age and nutrition of the host, season, temperature, and light, as well as virulence of the pathogens and resistance of the host (9, 29, 68, 69, 93, 97, 112, 119, 128, 137). The two types of symptoms in Panama disease, viz., petiole buckling without leaf yellowing and slow wilting of leaves after yellowing, are suggested to be due to difference in toxin production between pathogenic clones [Page (94)].

Whatever the range of pathological syndrome, a growing imbalance in water economy in the affected plants is the obvious symptom common to all.

Internal Symptoms

Most wilt pathogens grow in the xylem elements of their hosts and, by their diverse activities, contribute variously to water shortage and final collapse of the plants.

The physical presence of the pathogen in vessels.—Although this will normally be associated with disease, it is generally not considered a major cause of wilt, except perhaps in bacterial wilt (27). A sparse internal distribution of hyphae and conidia seems characteristic of hadromycoses. This is strongly suggestive of their metabolic products playing a major role in disease.

Occluding materials and vascular browning.—These include tyloses, gums, pith and xylem hyperplasia and hypertrophy, calcium pectate gels and other disintegration products of host pectic and cellulosic materials. Extensive tissue disintegration, prior to wilt, is noticed perhaps only in bacterial wilts. Although pectic and cellulolytic enzymes have been demonstrated to be active *in vivo* in these plants, their role is thought to stop with tissue degradation and promoting rapid distribution of the bacteria. A heat-stable viscous bacterial slime, with a distinctive reticulate pattern, believed to be a complex polysaccharide, appeared to be the actual cause of wilt, by increasing the viscosity of the vascular stream and interfering with water movement [Husain & Kelman (51, 52)]. The possible role of pectic enzymes of pathogens, acting on host cell walls liberating partially hydrolyzed gels, gums, and phenolic compounds which may contribute to vascular occlusion and browning, has been reviewed (27). It is clear that the various constituents, DP (depolymerase), PME (pectin methylesterase), and PG (polygalacturonase), have independent disintegrating properties. Nevertheless, whether any of these has a direct bearing on wilt seems problematical. Culture filtrates of a mutant strain of *Fusarium oxysporum* f. *lycopersici*, lacking pectolytic enzyme but containing PME, induced epinasty, yellowing, vascular browning, and even vascular plugging, yet the severe symptom of wilting did not occur in tomato shoots [McDonnell (88)]. PG would seem to be more involved in wilts and it has been detected *in vivo* in diseased plants (52, 109). It does not seem plausible, however, that PG could act without the complementary activities of PME and DP [Wood (136)].

Extensive tyloses and gums have been observed in many diseases, forming prior to wilting (115). Excellent direct correlation between such plugging, failure to conduct dye and radioactive solutions, and wilt symptoms has been found in oak wilt particularly [Parmeter *et al.* (97); Schoeneweiss (110)]. The wilt seems to result when the normally functioning outermost ring of springwood vessels also gets plugged with pathogenically induced symptomatic tyloses, which differ from the normal tyloses in older annual rings in being more numerous, tightly packed, and yellowish *en masse*. Esau's (31) report about tyloses in xylem vessels in Pierce's disease of grapes, where the vines sometimes show wilting and die-back, is interesting. These, as well as vascular or pith hyperplasia and hypertrophy, no doubt indicate the action

of growth substances which may be either of pathogenic origin or from pathogen-induced stimulation of host cells and can account for wilt. Talboys (130), however, considered tylose formation in hop varieties, controlled by a first "determinative phase," as contributing to resistance by excluding the pathogen from the stele. Probably the site and extent of tylose formation may be governed by the amount of growth substance produced, which will differ in particular host-parasite combinations.

There seems justification enough to consider vascular plugging, forming some time during pathogenesis, as a major factor leading to wilt. Even where these have not been noticed in fixed material, vascular occlusions were seen in fresh sections (9, 85). This is not to exclude the action of necrosis-inducing toxic metabolites of the pathogen, evidence for which is fast accumulating, particularly after the demonstration of toxins such as fusaric acid as true vivotoxins (36, 37, 95, 101, 127). After all, one has to bear in mind also that in the wilt syndrome, the major symptom of wilting and collapse is often accompanied by other varied symptoms such as epinasty, yellowing, etc. That toxic products of fungi alone can induce wilt is indicated by the wilting of peach and pear seedlings due to growth of a contaminating saprophytic species of *Penicillium* [Diener & Weaver (26)].

Morbid anatomy of leaves.—Severe plasmolysis of leaf cells, disintegration of plastids, as well as the occurrence of brown occluding materials at the vein-endings, have all been reported in the earlier stages of wilt, suggesting that the action takes place in advance of toxins, rendering the leaf cells ineffective in maintenance of the transpiration pull [Kalyanasundaram (58); Struckmeyer *et al.* (119); Talboys (127)]. The recent report of a rise in temperature coefficient of leaves, petioles, and stems of wilting watermelon plants, and the similarity of this response to phytonivein treatment, indicates a "plant fever" akin to a reaction in animal pathology (92).

DERANGEMENT IN HOST METABOLISM

Alteration in permeability.—It is clear from the foregoing that visual symptoms are preceded by and are the consequence of an alteration in the host metabolism. In vascular wilts perhaps the primary impact of the presence of the pathogen and its toxic metabolites is on the water relations of the host plant. Apart from the physical factors, which have been discussed in detail with reference to our current knowledge on the mechanism of the ascent of sap (124), toxic metabolites of the wilt pathogens have been implicated in the impairment of the water balance of the diseased plant. The effects of lycomarasmin, the first wilt toxin to be isolated, on the transpiration and absorption by tomato cuttings and its capacity to impair the semipermeability of tomato leaf cells and the repercussions on the water turnover and water balance of the host plants have been explicitly summarised earlier (27, 35). Gäumann & Naef-Roth (41) assume that the excessive transpiration occurring subsequent to lycomarasmin poisoning is mainly because of its

"vehicle effect" causing a "local iron plethora" in the leaves which results in iron injuries. This assumption has been verified using *Rhoeo* protoplasts (38). Lycomarasmic acid, a transformation product of lycomarasmin has been recently reported to induce qualitatively the same injury symptoms as lycomarasmin does. Its mechanism of action on tomato shoots is supposed to be analogous to that of lycomarasmin, though in the acid form it is thrice as active and has a stronger chelating capacity (42). Fusaric acid, because of its uneven distribution inside the host, causes localised transpirational disturbances leading to necroses of interveinal areas in the leaf. Chemical changes of fusaric acid such as decarboxylation increase toxicity; the disturbance to the water permeability of the protoplasts of the host cells caused by 3-*n*-butyl pyridine is about 100 times more than that caused by unmodified fusaric acid which acts as "protoxin" (6). Methylation of the nitrogen atom in conjunction with acid amide formation results in loss of the toxigenic characteristic of fusaric acid, rendering it unable to cause wilting of tomato shoots. Nevertheless, at lower concentrations its pyridine ring still causes excessive water permeability of the protoplasts (70). The threshold of impairment, by fusaric acid, of osmotic water uptake by medullary cells of young tomato plants is 10^{-7} to 5×10^{-8} *M* (40) which is easily attained both *in vitro* and *in vivo* (59, 63, 118). The damage to water permeability increases with increasing concentrations of fusaric acid up to 10^{-4} *M* beyond which permeability is not measurably impaired because of a "water proofing" effect attributable to coagulation of plasma. The configuration of the fusaric acid molecule is invoked to explain this change in the direction of the dosage effect relationship, which depends also on the plasma type of the test plant (6). Further, the reaction of the protoplasts to fusaric acid is altered by a change in the nutrition of the plants. For instance, overfeeding with nitrogen, phosphorus, and potassium increases the resistance of the protoplasts to the pyridine ring of fusaric acid. Underfeeding with K has no effect while N or P deficiency suppresses completely the sensitivity of *Rhoeo* protoplasts (39). Zähner (137) has reported that an increase in salt concentration in the medium increases resistance of tomato plants to fusaric acid, lycomarasmin, and its Fe-complex. Nitrogen deficiency strongly reduced the susceptibility of tomato to fusaric acid. This is probably further borne out by the fact that an increase in nitrogen nutrition increases susceptibility (23, 107). However, the alteration in susceptibility cannot be fully explained by a change in the response of the protoplasts to fusaric acid since parallel instances have been observed in other wilt diseases where fusaric acid is not known to be involved in pathogenesis (69).

Besides damaging the passive intake of water by protoplasts, fusaric acid also impairs the active absorption of water. Since this uptake requires energy liberated by respiration, it could be blocked by an inhibition of respiratory enzymes, especially cytochrome oxidase. Bachmann (6) reports that the effect of fusaric acid at the higher concentrations disappears when cyto-

chrome oxidase is inhibited by sodium azide. When KCN is used as the inhibitor, however, the water permeability affected by fusaric acid is not changed. This is rather surprising since the mechanism of action of cyanide and azide on the cytochrome-*c*—cytochrome oxidase system is not only similar (53) but also the complexes formed by cyanide, though reversible, are rather more stable than those formed by azide (49, 65). Presumably, the disturbance by fusaric acid of the nonosmotic water uptake by protoplasts is through an impairment of the semipermeability of the mitochondrial membrane so that cytochrome-*c* escapes and is thus removed from the site of action [Paquin & Waygood (96)]. The use of more specific inhibitors could probably lead to a better understanding of this aspect of fusaric acid injury.

Impairment of the semipermeability of the plasma membrane within a few hours after fusaric acid is administered to cut shoots of tomato is evidenced by a sharp increase in the cuticular excretions. Potassium ions, in a quantity up to 300 times greater than the controls, were released in addition to sodium and calcium ions, some amino acids, and peptides [Linskens (82)]. An increased conductivity recorded in *Fusarium vasinfectum*-infected cotton plants has been attributed to the accumulation of electrolytes due to primary changes in osmoregulation of the host cells (43). Spectrochemical analysis of infected cotton indicates a significant derangement in the ionic balance of these plants (102). In the leaves of infected plants there is a considerable decrease in Mg, Ca, and K, and a slight increase in Mn. The apparently healthy plants show a marked increase in Mn and Mg and a decrease in Ca and K, which indicates a derangement of permeability of the plasma membrane leading to a loss in the selective absorption of ions. The slight ionic imbalance exhibited by the resistant variety grown in inoculated soil may be due to the presence of the pathogen in the root region which is unable to establish itself; perhaps toxin production is prevented (103).

Changes in carbohydrate metabolism.—A high reserve of carbohydrates and a high ascorbic acid content are characteristic of the two strains of *Gossypium hirsutum* naturally resistant to *F. vasinfectum.* The susceptible strains of *G. arboreum* have a low ascorbic acid and carbohydrate reserve (56) that may, however, be modified to simulate the condition obtained in the resistant strains by feeding the plants with high concentrations of Zn or Mn (57). An increase in auxin level induced by a higher Zn content and perhaps a more effective utilization of available auxin by the otherwise naturally Zn-deficient Asiatic cottons (*G. arboreum*) are suggested to explain this improved plant vigor. A reduction in ascorbic acid and an increase in reducing sugars seen in *Fusarium*-infected cotton and red-gram plants (55), seems rectifiable by a Zn amendment of the soil (125). Susceptible cotton strains grown in Zn-amended soil show a reduction of pectic substrates in the roots (122) which, if present in higher amounts, make for susceptibility (77). However, the mechanism of wilt control effected by soil amendments with Mn (106) and Fe/Mn (134) is little understood as yet.

Changes in nitrogen metabolism.—Alterations have been reported in the

nitrogen status of wilting plants (107, 112). In *Fusarium*-infected cotton the net effect of infection seems to be a depletion in the total NPN content. This is attributed to a powerful oxidative deaminase system of the pathogen, whereas PN content is not altered to any significant degree; consequently, the ratio NPN/PN goes down in the infected suscept while in the resistant ones it remains the same even when grown in inoculated soil (76). An accelerated protein synthesis is also suggested to account for resistance since the much needed NPN, in the form of amino acids that go to form the toxic metabolites (104) responsible for the disease syndrome, is made unavailable to the pathogen. This is further substantiated by studies on the free amino nitrogen constituents of the different cotton strains where both qualitative and quantitative changes in the distribution of amino acids have been observed (80, 122). Cystine has been consistently detected in all parts of the resistant strains of cotton while the suscepts are devoid of it. This amino acid has been noticed to confer protection against fusarial toxins, presumably by preferential chelation with available iron which is known to potentiate the toxin(s) (75). It has been shown very recently, however, that cystine does not hold iron with such avidity as to render it unavailable for at least fusaric acid *in vitro* (86).

Respiratory changes.—Fusariose wilts cause a pronounced increase in O_2 uptake by the diseased tissues (22, 72). This remains essentially unaltered by varying total salt or N,P,K concentrations in the medium, except when plants are grown at very high nitrogen levels (108). The increase is preceded by a decrease in the early stages following infection (22, 72). This depression is probably caused by fusaric acid, which is known to inhibit tissue respiration of cotton and tomato (71, 89). Chelation with metallic components of metallo-enzymes (37, 131) is suggested as the probable mechanism of respiration inhibition and this possibility is supported by the fact that pre-chelation with some heavy metals makes the toxin less inhibitory to the respiration of cotton (71). The augmented rate of O_2 uptake that follows is perhaps caused by other metabolic products of the pathogens such as auxins, which are known to be produced *in vitro* (74).

Qualitative changes have not been observed in the respiration of diseased tomato plants (108). Nevertheless, in the case of cotton, a stimulation of the terminal oxidases is suggested since augmented respiration is almost completely inhibited by $M/500$ cyanide. Reports of increased ascorbic oxidase and polyphenol oxidase activity of diseased cotton (73) and an increase in the polyphenol oxidase activity of *Fusarium*-infected tomato plants (105) lend support to this view. Whether these changes are simply incidental in wilt pathogenesis, it is premature to say at present.

MECHANISM OF WILT

Without being unduly speculative we could summarize our ideas on pathological wilt based largely on our knowledge of the vivotoxin fusaric acid. Undoubtedly, this toxin can be formed rapidly in the rhizosphere

within 48 hr. of the fungus coming in contact with the root, and its absorption, decarboxylation, and increased toxicity *in vivo* can take place with equal rapidity. It appears that the first damage to tissues is impairment of water permeability, quickly followed by loss in conductivity of sap by about the sixth day of inoculation. Simultaneously, exaggerated transpiration resulting in loss of turgor and imbalance in the ionic status of affected cells occurs, with an almost total loss of potassium, and excessive exudation of electrolytes from leaves takes place. The other *in vivo* changes, such as increased respiration, hyperauxiny, reduction in total ascorbic acid, impairment of oxidative phosphorylation, inhibition of succinoxidase, and cytochrome oxidase activity of the mitochondria, all appear to be the sequelae of the earlier tissue damage. The question really is whether, in the intact rooted plant, without the pathogen entering the tissues, all these metabolic changes can be induced by the toxin generated by the pathogen in the rhizosphere alone. Quite obviously it can. We also have evidence showing that the utilization and enzymic hydrolysis of the pectin reserve by the pathogen in the roots, post-entry into tissues, can result in a sequence of events leading to vessel plugging. But whether, in this short time schedule of rapidly changing host physiology, this vessel plugging is penultimate or ultimate is a moot point.

The writer believes that the damage to tissues by toxaemia in the first few days is almost irrecoverable; and that the formation of gels in vessels resulting from the action of pectic enzymes and the tyloses which form due to hyperauxiny are secondary effects that seal the fate of the already crippled host metabolism and make the chances of recovery almost remote. As already stated, great interest will center on any future attempt to discovering fungal strains with little or no PME, PG, or DP, or that show poor ability to induce hyperauxiny *in vivo* and yet could grow and flourish in the rhizosphere and continue to produce optimum concentrations of toxin.

THE TASK AHEAD

In an earlier publication Horsfall & Dimond (50) stated: "For reasons of exactness of definition, it will be assumed that the fungus can cause disease from the moment it enters the host." This may necessarily have to be redefined in light of evidence now accumulating on the production of toxins and antibiotics in the soil and in the rhizosphere. However, any rigid definition of the beginnings of disease production would seem in jeopardy as increasing advances in techniques may permit detection of very minute changes in the metabolism of the root and shoot consequent to growth of the fungus in the rhizosphere. Maybe we will also succeed in detecting the elaboration of toxins in the rhizosphere and be able to correlate eventually with the all-important time factor, as a variety of physiological processes seems to be affected in the host even within the first few days of initiation of the disease and long before the development of the disease syndrome. The

questions really are: whether the production of the toxin in the rhizosphere is a continuous process; and whether the energy substances available for such spontaneous elaboration of the toxin would not diminish with the onset of toxaemia, and if so, would not penetration into root tissues by the fungus in search of new energy substrates be imperative? There may, after all, be a chain of events leading to wilt, of which we now know only a few. Unless and until we have techniques by which fungal growth and activity in the rhizosphere can be quickly arrested at will, after it has done its job of toxin production utilizing almost solely the available energy exudates in the rhizosphere, it would seeem difficult to adopt a firm line of thought. Quite obviously, such a fungal inactivation technique by either chemotherapeutants and/or antibiotics, or by radiations, has to be selective in so far as the growth of the microorganism is concerned without injuring the host root. There is another side to this question: toxic substances are known to be excreted by plant roots into the soil and these have proved inhibitory to plant growth. An excellent example of this is the trans-cinnamic acid, secreted by the guayule plant, which is highly toxic to guayule seedlings; even more interesting, guayule seedlings were at least a hundred times as sensitive to cinnamic acid as tomato seedlings [Bonner (12)]. The presence or absence of specific inhibitor(s) or toxic substances exuded by plant roots that are inimical to the growth and production of microbial toxins or antibiotics in the rhizosphere could, therefore, be usefully investigated. In short, any attempt at understanding the microecology of soils of the rhizosphere of plants susceptible to fungal wilts and the production and exudation of toxins by plants would repay itself as surer methods of breeding resistant varieties of plants to fungal wilts could then be employed.

While still considering the rhizosphere, we could briefly discuss the problem of adaptation of pathogens. Buxton (20) has covered this subject ably. There appears to be a *prima facie* case for facultative parasites, like causal agents of wilt, showing variation in their adaptive enzyme systems and consequently in virulence. These adaptations are known to be temporary in some cases and permanent and heritable in others. Most of the wilt-producing organisms belong to the Deuteromycetes, which are notorious for displaying heterokaryosis and saltation. Therefore, it is not uncommon to find in nature a whole series of forms ranging from complete avirulence to virulence.

Among the major problems in the study of the wilt syndrome is the question of substrate for the pathogen once it gains entry into the root. Here, the carbon and nitrogen reserves in the susceptible host have to be intensively studied. This study will also have to be extended to a screening of oxidative cellular changes, electrophoretic studies on protein changes, production of wound and growth hormones related to pathogenesis and the detection of vivotoxins where they exist. Also, the coupling and uncoupling mechanisms in respiration correlated with the production of vivotoxins have

to be better understood. The ionic status of susceptible and resistant plants to wilt, especially where the plants have a distinct genetic parentage as shown in the case of cotton [Sadasivan & Kalyanasundaram (102); Sadasivan & Saraswathi-Devi (103)], will have to be studied further; for example, that the total available amount of a heavy metal like iron could potentiate vivotoxins (37). The new concept of the presence of inhibitors to vivotoxins not only in the rhizosphere but *in vivo* also (62) has to be explored as it has potentialities of practical application in wilt control.

One of the most important metabolic derangements taking place in some wilt diseases appears to be a detectable change in the auxin status of affected plants. Hyperauxiny reported in tomato infected by *Verticillium albo-atrum* is perhaps the most authentic (98), although many authors have reported increased axillary bud activity after the wilt syndromes became apparent. Here, then is a case where studies on antiauxins would pay because the main mechanism of antiauxin effects presumes a binding of the auxins to a receptor of protein nature (the receptor proteins are now considered to vary from one organ to another in the same plant). It is well-known that auxin molecules exert their growth-regulating effects only when so bound to active sites; or it could also mean competition with auxin at the growth centers. Thus, the growth of shoot parts with suboptimum auxin levels would be retarded by application of an antiauxin which would seem to compete with native auxin for available active sites [Åberg (1)]. If this be granted, then in roots containing supraoptimum auxin levels this competition should lead to accelerated elongation. In systems like *Verticillium* wilt of tomato, where high IAA increases were recorded, i.e., it was saturated with an auxin presumably of high activity, there should be increased antiauxin effects, or conversely if the plant were poor in auxin content then the resultant effect would be a positive auxin effect. These and related problems commend themselves for experimentation.

There is considerable need for also working on the gibberellins in relation to auxin, especially in the *Fusarium moniliforme* foot-rot of rice where we have an elongation phenomenon ("bakanae" effect) or gibberellin effect and the fusaric acid syndrome coexisting. Indeed, a beginning has been made recently in this laboratory to follow up this problem [Andal (3)]. The results showed that the typical foot-rot plants had a lower rate of O_2 uptake than their healthy controls, whereas the "bakanae" plants had a higher O_2 uptake than their controls. The presence of fusaric acid in a greater quantity in tissues of the typical foot-rot plants was obvious when they were compared with the "bakanae" plants and this probably explains the retardation in respiration since the vivotoxin, fusaric acid, is known to have such an effect. In future studies more emphasis should be laid on respiratory substrates affected by foot-rot and "bakanae" conditions in order to throw light on *in vivo* interactions between fusaric acid and gibberellin and indicate whether there is any evidence of an uncoupling mechanism in respiration. Although

not a typical wilt disease, this foot-rot of rice offers many interesting experimental avenues for studying the interaction of gibberellins, IAA, wound hormones, and fusaric acid *in vivo*.

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AUTHOR INDEX

A

Aamodt, O. S., 270
Aasheim, T., 346, 355
Abadia, A., 76
Abdul-Nour, B., 123, 126, 127, 254
Abeles, F., 185
Aberg, B., 345, 464
Ackley, C., 431
Acs, G., 119, 120, 121
Adams, D., 107
Adams, G. A., 36
Adamson, A. W., 97
Adamson, D., 53, 54, 57
Adamson, H., 53, 54, 57, 397, 398
Adelstein, S. J., 72
Adler, E., 2
Afridi, M. M. R. K., 70
Agnihothrudu, V., 452, 453
Agranoff, B. W., 223, 226, 227
Ahmed, S., 64, 66
Akazawa, T., 19, 20, 28, 249, 251
Albaum, H., 196, 197
Alberghina, F., 209
Albersheim, P., 36, 37, 44, 54, 55, 56, 207, 210
Alcorn, J. W., 379
Alder, E. F., 371, 372
Aleem, M. I. H., 74
Alexander, M., 73, 74
Alfert, M., 330, 336
Allan, R. E., 380
Allard, H. A., 304
Allen, E., 115, 116, 120, 123, 125
Allen, E. H., 119
Allen, F. L., 178
Allen, M. B., 78, 122, 170 255
Allen, O. N., 398, 399
Allen, P. J., 81
Allen, S. E., 94
Allfrey, V., 128
Allison, F. E., 92
Allison, R. M., 3, 7
Allmendinger, D. F., 279, 280
Allsopp, A., 37, 355, 399, 400
Altermatt, H. A., 40, 43, 205
Altman, R. F. A., 347
Alvim, P. T., 282, 372, 377
Amann, H., 177
Ambe, K. S., 22, 23, 24, 25
Amer, F. A., 273
Amesz, J., 157, 185
Amin, J. V., 92
Andal, R., 464
Anderson, D. B., 268
Anderson, D. G., 199, 221, 222, 229, 231
Anderson, E., 37
Anderson, I., 80
Anderson, I. C., 238
Anderson, W. W., 26
Andersson-Cedergren, E., 14
Andreae, W. A., 347, 355, 356, 362, 456
Andreatta, C., 92
Andrews, F., 245, 247
Andreyeva, T. F., 277
Andrus, C. F., 454
Anhder, L. D., 379, 389
Anker, L., 410, 415, 416, 423
Antoniani, C., 92
Appel, E., 410, 424, 426
Appel, W., 177
Appleby, C. A., 22, 67
Applewhite, T. H., 278
ApRees, T., 202, 203, 214
Aragona, G., 347, 358
Arhimo, A. A., 8
Armiger, W. H., 98, 99
Armstrong, M. D., 345, 363
Arnold, W., 165, 169, 187, 188, 189
Arnold, W. N., 433
Arnon, D. I., 64, 65, 66, 78, 93, 178, 183, 186, 187, 196, 201, 207, 255
Aronoff, S., 158, 250
Aronson, J. M., 36, 58
Arora, N., 398, 399
Arrigoni, O., 202, 203
Arslan, T., 427
Arthur, W., 81
Asahi, T., 208
Asana, R. D., 282
Asano, A., 70
Ashcroft, R. T., 75
Ashida, J., 52, 53
Ashton, F. M., 279, 280, 377
Ashton, M., 58
Aslyng, H. C., 93, 97
Aspinall, G. O., 36, 40, 44
Atchison, A., 19, 20
Athanasiades, J., 360
Atkinson, D. E., 71
Atkinson, G. F., 452
Atkinson, M. R., 195, 200, 204
Audia, W. V., 376, 378
Audus, L. J., 349, 354, 410, 412, 418, 419, 420, 426
Austrian, R., 40
Avron, M., 178, 182, 183, 184, 187, 238
Axelrod, B., 43, 196, 199, 201, 202, 205, 210, 212
Axley, J. H., 92
Ayers, A. D., 280
Azimov, R. A., 84

B

Bachmann, E., 459
Bacon, J. S. D., 77
Baddiley, J., 118
Baeckeland, E., 337
Bailey, I. W., 38
Bailey, J., 245, 247
Bailey, L. F., 345
Bajer, A., 339
Baker, E., 44, 45
Baker, J. E., 21
Baker, K. F., 454
Bakhiv, A. I., 277, 281
Balashov, V., 36
Balce, L. V., 249, 254
Ball, C. D., 44
Ball, E. G., 27
Ballantine, J., 26
Ballard, J. C., 381, 385
Ballin, G., 355, 356, 363
Bamberger, E., 402
Bandhuin, P., 29
Bandurski, R. S., 38, 77, 201, 208, 414, 420
Banerjee, S., 347, 356, 358
Bange, G. G. J., 102, 106, 269, 272, 273
Bannarjee, S., 76
Bara, M., 415, 427
Barber, D. A., 107, 283
Barber, M. S., 219, 234, 235
Barbier, G., 94
Barbour, R. D., 256
Bard, R. C., 7
Barlassina, G., 201
Barr, R. A., 257
Bartholomew, E. T., 268
Bartlett, G. R., 196
Barton, L. V., 379
Barton, N. L., 99
Basch, S., 337
Basford, R. E., 27, 83
Bassham, J. A., 158
Bässler, K. H., 2
Bates, H. M., 19, 115, 116, 128, 251

Batty, J. E., 347
Baxter, P., 77
Bayley, S. T., 35-62; 36, 37, 38, 39, 42, 45, 46, 47, 48, 49, 51, 52, 54, 55, 56
Bazyrina, E., 141
Beaman, T. C., 38
Bear, F. E., 75, 77
Beauchamp, C., 348
Beaufay, H., 28, 29
Beckman, C. H., 456, 458
Bedri, A., 312
Bedri, A. A., 77
Beer, M., 38, 42, 45, 46, 47, 48, 49
Beer, R., 48
Beevers, H., 19, 20, 28, 43, 83, 196, 199, 202, 203, 205, 207, 210, 212, 214, 249, 250, 251, 256
Behrens, M., 29, 347
Beiler, A., 142
Beimler, R., 350, 353, 354
Beinert, H., 23, 27, 83
Belcher, R., 77
Beling, H., 378, 389
Beling, I., 304
Beljakoff, E., 142
Beljanski, M., 114, 129
Bell, F. H., 143
Bell, H. K., 374
Bell, L. N., 160
Bendall, D. S., 16, 20, 21, 29, 76, 83, 214
Bendall, F., 177
Benedict, H. M., 246
Benfield, A. H., 255
Bennet-Clark, T. A., 39, 53, 54, 77, 350, 353, 355, 414, 426, 427
Benson, A., 158
Bentley, J. A., 345, 346, 350, 351, 353, 354, 355, 356, 357, 361
Berenblum, F., 196
Berg, A. -M., 4, 8
Berg, P., 118, 119, 120, 121, 208
Berg, V., 141
Berge, G., 147
Bergeron, J. A., 237, 238
Bergmann, F. H., 118, 119, 120, 121
Bergqvist, G., 378
Berman, H., 92
Bernheimer, H. P., 40
Bernlohr, R. W., 119, 247
Bernstein, L., 97, 99, 283, 378
Bershova, O. I., 66
Bertheux, M. H., 93
Bertrand, D., 64, 81
Best, R. J., 456
Beth, K., 254
Bewley, W. F., 456, 458
Beyer, R. E., 26
Bhuvaneswari, K., 452, 453
Biale, J. B., 77, 247, 248, 250, 255, 256, 257, 259
Bianchetti, R., 198, 199, 205, 212, 213
Bibring, T., 339
Bickle, S., 355
Biddiscombe, E. F., 371
Biebl, R., 143, 246
Bierhuizen, J. F., 269, 286
Bieri, J. G., 25
Biggar, J. W., 95
Bilan, M. V., 377
Bils, R. F., 18, 19, 20, 28, 251
Bird, L. S., 379
Bishop, C. T., 36, 37, 38, 39, 54
Bishop, J., 123, 125
Bishop, N. I., 25, 238
Bishop, O. W., 100
Bitancourt, A. A., 345, 346, 359
Bjorksten, J., 245, 247
Blaaw-Jansen, G., 348
Black, J. N., 137, 145, 149
Black, M., 381
Blackman, F. F., 133, 134
Blackman, G. E., 137, 145, 149
Blagowestschenski, W. A., 139, 140
Blair, G. Y., 95
Blair, J. M., 135
Blaney, L. T., 307
Bleasdale, J. K. A., 436
Blinks, L. R., 163, 171, 172, 173, 174, 177, 237
Bloch, D., 330
Bloch, K., 224, 259
Bloch, R., 451
Blom, J., 6
Blommaert, K. L. J., 345, 346, 352
Bloom, B., 256
Blumenthal-Goldschmidt, S., 351, 400, 406
Blust, G., 433
Boawn, L. C., 94
Bobrov, R. A., 435, 442
Bocchi, A., 318, 399, 406
Block, R. M., 127
Bode, H. R., 422
Boeri, E., 22
Bogorad, L., 310, 319
Böhmer, H., 44, 45, 47, 49
Böhning, R. H., 134, 135, 144
Boken, E., 81
Bolliger, R., 46, 49
Boman, H. G., 119
Bomstein, R., 22
Bond, G., 66
Bond, V. P., 328
Bonde, E. K., 346, 354
Bone, D. H., 26
Bonner, B. A., 297, 298
Bonner, D. M., 400, 402
Bonner, J., 36, 37, 38, 39, 44, 45, 47, 49, 52, 53, 54, 55, 56, 57, 113, 125, 128, 207, 210, 222, 229, 250, 251, 253, 254, 255, 271, 274, 277, 278, 281, 293, 294, 295, 299, 300, 306, 308, 310, 311, 313, 314, 315, 316, 317, 318, 347, 361, 383, 463
Boodley, J. W., 376
Boon-Long, T. S., 274
Boonstra, A. E. H. R., 141
Booth, A., 350, 354
Bordeau, P. F., 134, 135, 140, 142, 144, 382
Borland, J. W., 94
Bormann, F. H., 138, 148
Boroughs, H., 55
Borsook, H., 116
Borthwick, H. A., 293, 294, 296, 297, 298, 302, 303, 304, 307, 308
Bosian, G., 134, 141
Bossi, R., 454
Böszörményi, Z., 101, 102, 106
Bottelier, H. P., 410, 420
Bottomley, W., 403
Bould, C., 75
Boulter, D., 78, 82
Bouman, J., 25
Bourliere, F., 245, 247
Bovard, F. C., 120
Bové, J., 65, 128, 255
Bowden, K., 347
Bower, C. A., 93, 95
Bowler, E., 44, 45
Bowmann, H. G., 120
Boyd, G. E., 97
Boyer, P. D., 13, 119
Boyle, F. P., 185
Boysen-Jensen, P., 48, 51, 133, 134, 135, 136, 137, 138, 141, 144, 146, 147, 148, 422
Brachet, J., 246, 253, 254, 333, 334, 337
Brackett, F. S., 163, 174
Bradbeer, C., 66, 196, 207, 208, 249
Bradford, W. W., 381, 382
Bradley, M. V., 353, 354, 386
Bragonier, W. H., 456
Brain, E. D., 428
Braithwaite, G. D., 220, 222, 223, 225, 226, 231, 234
Brand, L., 19
Brandt, I. K., 128
Brauer, I., 335
Braun, A. C., 340
Braun, W., 401

Braunshtein, A. E., 7
Bray, J. R., 136
Bray, R. H., 95
Brecher, G., 328
Brennan, E., 441
Bretz, C. F., 320, 387
Brewer, R. F., 431, 433, 435, 437, 440
Breyhan, T., 354
Brian, P. W., 252, 347, 369, 380, 382, 383, 385, 386, 398, 405, 449
Bridgers, W. F., 19, 20
Briese, K., 157
Briggs, G. E., 99
Brilliant, B., 279
Brim, C. H., 372, 378
Britton, G., 351, 354
Brodie, A. F., 26
Brody, M., 155, 156, 170, 171
Brody, S. S., 169, 170, 187
Bromfield, S. M., 81
Bronchart, R., 345, 348, 349, 351, 356
Brougham, R. W., 145, 146
Brouwer, R., 100, 101, 107
Brown, A. H., 163, 185
Brown, A. M., 178
Brown, A. V., 114, 121, 122
Brown, B. G., 347
Brown, D. H., 41
Brown, D. M., 122
Brown, F. A., Jr., 307, 308
Brown, G. L., 114, 121, 122
Brown, H., 273
Brown, J. C., 75, 76
Brown, J. S., 172
Brown, S. A., 40, 43
Brown, S. W., 338
Brown, T. E., 81, 158
Brown, W. H., 274, 275
Brownbridge, M. E., 410, 412, 418, 419, 420, 426
Broyer, T. C., 64, 278
Brauner, L., 409, 410, 412, 415, 422, 423, 424, 426
Bruce, V. G., 304, 305, 307, 308, 309
Bruckbauer, H., 348
Bruemmer, J. H., 24
Brugger, J. E., 81
Brumfield, R. T., 410, 414, 418
Bryan, W. H., 38, 54
Bublitz, C., 221
Büchsel, R., 424
Buckley, W. R., 454
Buffel, K., 37, 54, 55
Buhler, D. R., 256, 259
Buhr, H., 134, 135, 141
Bukovac, M. J., 369, 370, 371, 374, 375, 376, 378, 379, 380, 381, 382, 383, 384, 385, 386, 388, 389
Bulard, C., 347
Bullough, W. S., 334
Bünning, E., 300, 305, 306, 307, 308, 309
Bünsow, R., 312, 318, 385
Burd, J. S., 93
Burg, S. P., 248, 257, 258, 259
Burgess, P. S., 93
Burgess, W. R., 268, 269
Burk, D., 103
Burk, L. G., 373
Burkina, Z. S., 276
Burnside, C. A., 134, 135, 144
Burris, R. H., 3, 7, 22, 66
Burström, H., 37, 52, 55, 77, 253, 278, 350, 417
Burt, L. M., 209, 215
Busch, S., 254
Busse, M., 52, 56, 57
Butenko, R. G., 318, 406
Butler, J. A. V., 125, 127
Bütler, R., 220
Butler, W. L., 296, 297, 298
Butt, W. D., 74
Buvat, R., 320
Buxton, E. W., 453, 463
Byerrum, R. U., 44, 207

C

Cabib, E., 44, 195
Cain, J. C., 375
Calhoun, B., 83
Calvin, M., 158, 187
Campbell, C. W., 311
Campbell, P. N., 113, 116, 129
Campbell, R. C., 374
Candela, M. I., 70
Cann, G., 432
Canning, R. E., 107
Cantoni, G. L., 121, 207
Cardiff, E. A., 434, 436, 439, 441, 444
Cardini, C. E., 40, 195
Carlier, A., 37, 54, 55
Carlson, J. G., 336
Carlson, R. M., 99
Carlström, D., 42
Carlton, A. B., 64
Carnahan, J. E., 7, 65, 66
Carpenter, W. D., 250, 256
Carr, D. J., 54, 305, 306, 316, 317, 318, 319, 356, 383
Cartellieri, E., 138, 139, 140, 141
Cartwright, P. M., 347
Caso, O. H., 384
Castelfranco, P., 119
Castle, E. S., 47, 49, 50
Castle, J. E., 7, 65, 66
Castor, L. N., 22
Cathey, H. M., 369-94; 303, 369, 370, 371, 376, 377, 378, 380, 381, 385, 387, 389
Cederstrand, C., 171, 173 174, 176, 238
Chailakhyan, M. Kh., 294, 312, 318, 386, 406
Chain, E., 196
Chakravarti, S. C., 383, 384
Chalmers, R., 171, 173, 174, 238
Chan, A. P., 319
Chance, B., 14, 21, 22, 23, 161, 165, 166, 168, 189, 199, 202, 211, 214
Chandhri, M. B., 92
Chandler, B. V., 222
Chandler, C., 379
Chang, S. C., 93
Chapman, H. W., 135, 138, 139, 140
Chappell, J. B., 17, 18
Chargaff, E., 126
Chastain, J. A., 99
Chayen, J., 255
Chaykin, S., 224, 259
Chattopadhyay, H., 347, 356, 358
Cheadle, V. I., 266
Chen, C. F., 95
Chen, P. T., 196
Chen, S. L., 237
Chen, Y. T., 82
Cheng, S.-C., 78
Cheniae, G. M., 26, 70
Cheong, L., 337
Cherry, J., 371
Chèvremont, M., 18, 337
Chèvremont-Comhaire, S., 337
Chibnall, A. C., 250
Chichester, C. O., 221, 222, 225, 236
Chichuk, A., 371, 372
Childers, N. F., 134, 136, 147, 148, 279, 281, 282
Ching, F. T., 373
Ching, T. M. T., 414, 420
Chinoy, J. J., 358, 363
Chmora, S. N., 144, 145, 146
Cholnoky, L., 235
Chopra, C. H., 14
Chouard, P., 293, 312
Chow, C. T., 250
Christiansen, G. S., 37, 52, 54, 56
Chung, C. W., 70, 71
Cifonelli, J. A., 40
Ciotti, M. M., 82
Claes, H., 232, 236, 306
Claesson, S., 162
Clark, C. S., 52
Clark, J. A., 278
Clark, J. E., 77, 345

Clark, J. S., 93
Clark, W. S., 148
Clarke, A. J., 361
Clarke, G. C., 345, 347, 348, 350, 355
Clarke, G. L., 142
Clarke, I. D., 20
Clay, E. I., 143
Claybrook, J. R., 400, 402, 406
Clayton, G. D., 431
Clayton, R. K., 165, 188, 189, 237
Cleland, R., 44, 47, 52, 53, 54, 55, 56, 57, 77, 210, 278
Clifton, C. E., 362
Clor, M. A., 387
Clowes, F. A. L., 329
Cocking, E. C., 255
Coggins, C. W., Jr., 375
Cohen-Bazire, G., 161, 229, 234, 239
Cohn, G. L., 128
Cohn, P., 125, 127
Colby, W. G., 107
Cole, C. V., 93, 95
Cole, J. S., 454
Cole, R. D., 118
Coleman, J. W., 162, 165, 166
Coleman, N. T., 76, 77
Coleman, R. E., 373
Collander, R., 106
Collins, R. P., 457, 461
Collins, W. T., 311, 314, 315
Colmano, G., 156
Colon, A., 329, 337
Colvin, J. R., 41, 42, 46, 47, 48
Comfort, A., 245, 247
Commissiong, K., 76, 79
Commoner, B., 158
Conn, E. E., 202
Conn, W. E., 196
Conover, T. E., 23
Conrad, C. M., 37
Constantzas, N., 362
Contopoulo, R., 186
Cook, J. A., 375
Cook, M. G., 92
Cook, W. S., 459
Coombe, B. G., 373, 386
Coombe, D. E., 139, 143, 149
Coon, M. J., 222
Cooper, C., 14, 25
Cooper, F. P., 46, 47, 48
Cooper, J. P., 380
Cooper, L. H. N., 147
Cooper, O., 27
Cooperstein, S. J., 23
Coote, J. L., 19, 28, 118
Corey, R. B., 93
Cormack, R. G. H., 54
Cormier, M. J., 119
Cornforth, J. W., 223, 227
Cornforth, R. H., 223
Corns, W. G., 371, 384
Corwin, L. M., 17, 18, 25
Coulombe, L. J., 377
Coursen, B. W., 452
Cowre, L., 205
Craddock, J. C., 380
Crady, E. E., 361
Craft, C. C., 258
Crafts, A. S., 265, 267, 435
Craigie, J., 205
Cralley, L. V., 431
Crane, F. L., 13-34; 13, 16, 23, 24, 25, 27, 28
Crane, J. C., 353, 354, 374, 386
Crane, R. K., 197, 209
Crane, S. M., 209
Crawford, C. R., 94
Cresswell, C. F., 72
Crick, F. H. C., 113, 124
Crickard, R. G., 163, 174
Criegee, R., 433
Cronkite, E. P., 328
Cronshaw, J., 35, 45, 46, 47, 49
Cross, B. E., 369
Cruickshank, D. H., 255
Csáky, T. Z., 4, 5
Cseh, E., 101, 102, 106
Cumming, B. G., 349
Currie, R. I., 145, 146
Currier, H. B., 265, 267, 387
Curtis, H. J., 246
Curtis, J. T., 142
Cutler, H., 348, 349
Cutolo, E., 22
Cutter, E. G., 321
Cuzzocrea, G., 347, 358

D

Daines, R. H., 441
Dalberg, F. W., 80
Dalgliesh, C. E., 113
Dallam, D. R., 26
Damaschke, K., 174
Dana, J. D., 92
Danckwardt-Lilliestrom, C., 398
Dandliker, W. B., 345, 346
Daniel, L., 84
Danielson, R. E., 283, 285
Dannenburg, W. N., 346, 355, 361, 362
Darkis, F. R., 282
Darley, E. F., 431, 432, 433, 434, 435, 439, 440, 441, 444, 445, 446
Darlington, C. D., 328
Darwin, J., 29
Das, N. B., 2
Das, N. K., 336, 338, 397
Das, V. S. R., 28
Dass, D. M. S., 14, 15
Dastur, R. H., 279, 280
Davenport, G. E., 22
Davenport, H. E., 76, 78, 165, 185
Davenport, J. B., 257
Davern, C. I., 332
Davidis, U. X., 373
Davidson, H., 382
Davidson, R. S., 400
Davie, E. W., 119
Davies, B. H., 222, 237
Davies, D. D., 204, 205, 208
Davies, E. B., 92
Davies, R. E., 16
Davis, A. R., 269, 271
Davis, D., 376, 456
Davis, E. A., 451
Davis, H., 135, 141
Davis, J. W., 118, 207
Davis, L. E., 95
Daxer, H., 143
Day, P. R., 98, 99
De, D. N., 330
Deal, P. H., 370
Dean, L. A., 92, 93, 94, 283
DeBernard, B., 23
de Bools, H. M., 410, 420
Decker, J. P., 134, 139
deDuve, C., 15, 16, 28, 29
Degman, E. S., 280, 283
Dekker, E. E., 222
DeKock, L. L., 246
DeKock, P. C., 75, 76, 77 246, 256, 257
Del-Campillo, A., 222
De Ley, J., 6
Delouche, J. C., 381
Demain, A. L., 129
DeMoss, J. A., 118, 119
DeMumbrum, L. E., 92
Denecke, H., 140
Denmead, O. T., 282
DeRoo, H. G., 441
deRopp, R. S., 396, 400, 401, 405
DeTurk, E. E., 95
Deuel, H., 107, 356
Deul, D., 25
de Vazquez, E. M. S., 356
Devlin, T. M., 14, 25
De Vries, J. E., 346
De Waard, A., 224, 259
de Wit, C. T., 136, 148, 284, 285, 286
de Wit, J. L., 410, 415, 425
DeWolf, A., 81
Deysson, G., 398, 400, 401
de Zeeuw, D., 311, 313, 318
Dhariwal, A. P. S., 92
Dick, J. B., 452

Dickerson, A. G. F., 251
Dieckmann, M., 119, 120, 121
Diehl, A., 142
Diener, T. O., 458
Dijkshoorn, W., 100
Dils, R. R., 26
Dimond, A. E., 449, 450, 454, 456, 457, 458, 462
Dintzis, H., 125
Dintzis, H. M., 116
Dirks, H. T., 268
Di Somma, A. A., 208
Disselkamp, C., 81
Dituri, F., 221
Dixon, L. F., 282
Dixon, M., 104, 105, 245, 247
Djordjevic, B., 337
Dobbs, V. W., 359
Doeg, K. A., 23
Dolk, H. E., 415
Domanskii, R., 282
Domingo, C. E., 282
Domingo, W. E., 295
Donaldson, K. O., 25
Done, J., 8
Doneen, L. D., 286
Doney, R. C., 75
Donn, A., 338
Donoho, C. W., Jr., 381, 383
Doorenbos, J., 293, 295, 299, 303, 306, 312, 313, 317, 318, 382, 383, 385
Dorfman, A., 40
Dorresteyn, R., 186
Dostal, R., 295
Doty, M. S., 141
Doudoroff, M., 186
Dougall, D. K., 253
Douglas, J. R., 379
Douglas, T. A., 117
Downs, R. J., 296, 297, 303, 304, 387, 441
Doyle, W. R., 256
Drake, M., 107
Drautz, R., 141
Drew, R. M., 328
Drinker, P., 431
Ducet, G., 196, 209, 214
Duckworth, R. B., 46
Dugger, W. M., Jr., 83, 256
Dulbecco, R., 341
Dunn, D. B., 121, 404
Durr, I. F., 223
Durrel, J., 207
Dutton, H. J., 237
Duysens, L. N. M., 157, 160, 165, 166, 168, 169, 170, 177, 185, 237
Dworkin, M., 239
Dye, M. H., 345, 347, 348, 350

E

Eadie, G. S., 95, 104
Eakin, R. E., 399, 404
Earl, J. M., 256, 259
Earley, E. B., 371
Eaton, F. M., 93, 280
Eberhardt, F., 247
Edelman, J., 40
Edgington, L. V., 456
Edmondson, W. T., 136, 142
Edsall, J. T., 95, 104
Egami, F., 5, 66, 67, 69, 72
Eggerer, H., 223, 224, 226, 227, 259
Eggers, V., 347
Ehrlich, B., 25
Ehrmantraut, H. C., 165, 169
Eichenberger, E., 203
Eichhorn, G. L., 309
Eidinoff, M. L., 337
Ekdahl, I., 54
Elgabaly, M. M., 107
Eliasson, L., 213
Elifolk, N., 1
Elliot, W. H., 4, 73
Elliott, D. C., 78, 83
Elliott, W. B., 250
Ellis, B. G., 94, 95
Ellis, S. C., 257
Elson, D., 126
Elze, D. L., 272
Emerson, R., 155, 165, 169, 170, 171, 172, 173, 238
Emsweller, S. L., 379
Enderle, W., 269
Engel, E. K., 155
Engel, M. S., 73, 74
Engelbrecht, L., 402
Engelhard, A. W., 456
England, D. J. F., 251
Ensgraber, A., 279
Epstein, E., 99, 100, 101, 102, 103, 104, 106, 107
Erbland, J., 27
Ergle, D. R., 280, 378
Erickson, C. J., 379
Erickson, L. C., 438, 441, 445
Erickson, R. O., 334
Erkama, J., 66, 67
Ernster, L., 18, 23, 26, 196, 213
Esashi, Y., 385
Esau, K., 50, 266, 319, 459
Escombe, F., 273
Esnault, R., 414, 426, 427
Esposito, R. G., 65
Ettlinger, M. G., 4
Evanari, M., 351
Evans, G. C., 140, 143
Evans, H. J., 21, 23, 26, 63, 64, 66, 69, 70, 76, 80
Evans, L. T., 296
Evans, M. B., 238
Evenari, M., 381, 400, 406
Ewing, E. C., 381, 382
Eyster, H. C., 81, 158

F

Faith, W. L., 431, 446
Fang, S. C., 423
Farmer, V. C., 76
Farrar, K. R., 345, 346, 350, 351, 355, 361
Fawcett, C. H., 345-68; 77, 346, 350, 355, 356, 359, 361, 362
Fawcett, J., 14
Feingold, D. S., 40, 41, 43, 44, 195
Ferchau, H. A., 142
Ferenczy, L., 349, 352, 360, 363
Fergus, C. L., 456
Ferguson, J. J., Jr., 221, 223
Ferri, M. G., 361
Fewson, C. A., 66, 68, 71
Fiegelson, M., 16
Fieldes, M., 92
Fielding, A. H., 65
Fife, J. M., 316
Filfus, J. A., 230
Filzer, P., 134, 135, 139, 141, 142, 144
Finck, A., 92
Fine, J. M., 379
Fink, J., 17
Fireman, M., 95
Firket, H., 337
Firshein, W., 401
Fischer, A., 345, 347, 351, 352
Fisher, D. G., 36
Fisher, D. J., 66
Fisher, E. A., 97
Fisher, E. G., 70
Fisher, J. E., 303, 313
Fisher, M. E., 19, 20, 28
Fisk, E. L., 319
Fiskel, J. G. A., 92
Fitts, J. W., 95
Flaig, W., 354
Flemion, F., 381
Fletcher, W. W., 379
Florell, C., 19, 100
Fogg, G. E., 64, 136
Fogle, H. W., 381, 382
Folkers, K., 222
Folkes, B. F., 3, 196, 207, 250
Folkes, J. P., 114, 129
Fonnesu, A., 16
Foote, M., 250
Forsyth, F. R., 250, 252
Forsythe, W. M., 98, 99
Forti, G., 18, 20, 29, 183,

197, 198, 203, 210, 214
Foster, R. E., 455
Foster, R. J., 357
Fowden, L., 8
Fox, J. E., 397
Franck, J., 177, 178
Franklin, R. E., Jr., 107
Fransson, P., 349, 350, 353, 356, 417, 428
Fraser, D. A., 268
Fraser, P. E., 7
Frederic, J., 18
Fredericq, H., 313
Frederiks, J. C., 356
Freebairn, H. T., 442, 445
Freeland, R. O., 139, 141
Freiberg, S. R., 257
French, C. S., 25, 170, 172, 173, 177, 180, 238
French, S. H., 378
Frenkel, A., 155, 165, 166, 186, 240
Frenster, J. H., 128
Frenzel, B., 134
Freyre, R. H., 380
Frey-Wyssling, A., 35, 36, 38, 44, 45, 48, 49, 274
Fried, M., 91-112; 93, 94, 95, 96, 99, 100, 101, 102, 103, 106, 107, 122
Friedkin, M., 328
Friend, D. J. C., 303
Friend, J., 255
Fries, N., 399, 401, 404
Fritz, G., 203
Frondel, C., 92
Fujihara, F., 385
Fujimori, E., 162
Fujiwara, A., 64
Fukamizu, O., 314
Fukuharu, N., 377
Fukui, H. N., 345, 346, 348
Fukumoto, S., 385
Fuller, K. W., 58
Fuller, R. C., 238
Fuller, R. G., 400
Funk, H. S., 66
Furness, F. N., 63
Furr, J. F., 283
Furuta, T., 381

G

Gaarder, T., 133
Gaastra, P., 136, 137, 145, 279
Gabrielsen, E. K., 142, 146, 148
Gaffron, H., 155, 180
Gage, R. S., 267
Gail, F. W., 133
Gainey, P. L., 92
Gale, E. F., 114, 128, 129
Gall, H. J. F., 253
Gall, J., 331
Galston, A. W., 38, 48, 51, 80, 345, 356, 357, 359, 363, 379
Galun, E., 379
Gamble, J. L., Jr., 14, 25
Ganje, T. J., 93
Garber, M. J., 434, 437, 441, 445
Garbus, J., 19
Gardner, F. P., 295, 372
Gardner, W. R., 95, 283
Garner, W. W., 304
Garrard, E. H., 66
Garrett, R. H., 25
Garrett, S. D., 451, 454
Garton, G. A., 231
Gates, C. T., 277, 281, 282, 283
Gatt, G., 211
Gattung, N. W., 156
Gauch, H. G., 83, 284
Gaulden, M. E., 336
Gäumann, E., 449, 458, 459, 461, 464
Gaur, B. K., 20, 210, 212, 371
Gebhard, K. L., 246
Gedroiz, K. K., 94
Geleick, H., 157
Gelfant, S., 334
Genuth, S. M., 119
Gerloff, G. C., 66
Gerretsen, F. C., 66
Geschwind, I. I., 330
Gessner, F., 133, 134, 135, 139, 140, 142, 143, 145, 270
Gest, H., 165, 166, 240
Gey, K. F., 221
Ghosh, B., 347, 358
Gibbs, M., 196, 199, 202, 203, 256
Gibbs, M. H., 221, 222, 223
Gibbs, R. D., 268
Giever, P. M., 431
Gifford, E. M., Jr., 255, 320
Gilder, H., 69
Gilfillan, J. L., 222
Gilmour, H. S. A., 185
Gindel, I., 248
Gingrich, J. R., 267, 280, 286
Ginsburg, V., 40, 43
Ginzburg, B., 37, 39
Ginzburg, B. Z., 38, 39
Giovanelli, J., 259
Gist, G. R., 279
Giuditta, A., 23
Glaser, L., 41, 209
Glassman, E., 120
Glasziou, K. T., 38, 54, 252, 253, 398, 401
Gleason, L. S., 135
Gledhill, V. H., 283
Glenn, J. L., 16, 24, 26
Glenn, R. C., 93
Gloor, V., 25
Glover, J., 226, 235
Gnanam, P., 460
Godman, J., 330
Goedheer, J. C., 168, 237, 238
Goertzen, J. O., 95
Goldacre, P. L., 403
Goldberg, S. S., 92
Goldberger, R., 22
Goldman, D. S., 2
Goldstein, J., 120
Goo, M., 380
Good, N. E., 181, 347, 355, 356, 362
Goodman, T., 77
Goodwin, T. W., 219-44; 220, 222, 223, 224, 225, 226, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237
Gordon, J., 114, 121, 122
Gordon, S. A., 176, 345, 350, 355, 359, 360, 361
Gorham, E., 136
Gorter, C. J., 84, 356, 425
Gorton, B. S., 399, 404
Gothoskar, S. S., 457
Gott, J. W., 167
Gotterer, G. S., 18
Gottschall, V., 252
Govindjee, R., 172, 174, 175, 176, 185
Grabowski, A. V., 117
Gradmann, H., 271
Graham, A. F., 332
Graham, E. R., 94
Grahm, L., 421
Gran, H. H., 133
Granick, S., 69, 161
Gray, C. T., 26
Gray, L. H., 336
Gray, R. A., 81
Greathouse, G. A., 40, 41
Green, D. E., 13, 14, 15, 16, 22, 23, 24, 25, 26, 27, 66, 83
Green, J. G., 381
Green, M. C., 320
Green, P. B., 45, 46, 48, 50, 52
Greenberg, J. B., 345, 359, 363
Greenblatt, C. L., 155
Greenburg, L., 431
Greengard, O., 116
Greenlees, J., 23, 24
Greenridge, K. N., 267
Greenwood, E. A. N., 64, 66
Grégoire, V., 320
Gregory, F. G., 149, 272, 273, 275
Greulach, V. A., 305,

312, 317
Greville, G. D., 17, 18
Gribanov, V. M., 66
Griffin, D. H., 357
Griffioen, K., 37
Griffith, M. M., 398, 406
Griffiths, M., 161, 229, 239
Griffiths, S. K., 18, 20, 21, 209, 214
Griggs, W. H., 374
Grimm, P. W., 81
Grob, E. C., 220, 222, 223
Gromet, Z., 41
Gross, J., 116
Gross, P. M., 282
Grossenbacher, K. A., 269
Grossi, L. G., 123
Grove, J. F., 369
Grover, R., 358
Gruen, H. E., 345, 347, 348, 451
Grun, D., 331
Grünberger, D., 349, 361, 362, 363
Guern, J., 345, 346, 353
Guernsey, F. S., 313
Guerra, F., 17
Guha, B. C., 347, 357, 358
Gundersen, K., 386
Gunning, B. E. S., 354
Günther, G., 2
Gurin, S., 221
Gusev, N. A., 269, 270
Gustafson, F. G., 374, 375
Gustafson, J., 19
Guttman, R., 338, 401
Guttridge, C. G., 310, 385
Györgyfi, K., 235

H

Haagen-Smit, A. J., 345, 346, 400, 402, 432, 434, 439, 440, 443, 446
Haas, D. W., 18, 20, 21, 209, 214, 215
Haas, V. A., 158
Haber, A. H., 338, 377, 378, 387, 397, 400, 404, 406
Hackett, D. P., 13, 15, 18, 20, 21, 22, 23, 26, 82, 203, 209, 210, 214, 215, 247, 251, 257
Hacskaylo, J., 376
Haddock, J., 281
Haesloop, J. G., 312
Hagan, R. M., 265-92; 270, 279, 280, 281, 282, 285, 286
Hagberg, A., 380
Hagen, C. E., 94, 95, 99, 100, 101, 102, 103, 106
Hager, A., 415, 423
Haggman, J., 67
Hakala, M., 6
Halbsguth, W., 428
Hale, V. Q., 75
Halevy, A. H., 370, 371
Hall, A. G., 158
Hall, D. O., 77
Hall, N. S., 69, 94
Hall, R. H., 396
Hall, W. C., 450, 456
Hallaway, M., 250, 252
Hallsworth, E. G., 64, 66
Halsey, D., 373
Halvorson, H., 250
Hamann, A., 140
Hamilton, R. H., 414, 420
Hammar, C. -H., 2
Hammond, B. L., 380
Hamner, K. C., 44, 45, 293, 300, 302, 307, 308, 310, 311, 312, 313, 316, 317, 318, 319
Handley, R., 100, 102, 106
Hansen, B. A. M., 350
Hansen, E., 259
Hansen, H. N., 454
Hansen, V. K., 135, 144
Hanson, J. B., 18, 19, 20, 24, 27, 28, 99, 100, 102, 107, 251, 251, 252
Hanst, P. L., 433, 444
Harada, H., 313
Harada, T., 92
Harder, R., 134, 138, 139, 141, 142, 144, 312, 318
Harding, C. V., 338
Harding, R. B., 93
Harmon, J. W., 16
Harrington, J. F., 303, 375, 383, 384
Harris, D., 15
Harris, H., 332
Harris, P. J., 337
Harrison, A. L., 452
Härtel, O., 133, 136, 143
Hartenburg, W., 139, 141
Hartmann, G., 119, 120
Hartree, E. F., 22, 78, 460
Hartwig, E. E., 372, 378
Harvey, H. W., 147
Hase, E., 147
Hashimoto, T., 370, 380
Haskins, F. A., 78
Hassid, W. Z., 40, 41, 43, 44, 195, 253
Hatakeyama, I., 52, 53
Hatch, M. D., 212, 213, 250
Hatcher, J. T., 93, 95
Hatefi, Y., 19, 24, 25
Haupt, W., 310, 409
Hauser, E. W., 387
Hausermann, A. N., 272
Hawthorne, H. A., 283
Haxo, F. T., 141, 172, 174, 177
Hayashi, T., 378, 379
Hayward, H. E., 283
Hazeman, R. H., 201
Heald, W. R., 95, 96, 106
Healy, W. B., 78
Heath, O. V. S., 77, 149, 272, 274, 345
Hecht, L. I., 120, 121, 123
Hegarty, M. P., 257
Heggestad, H. E., 441
Heilbrunn, L. V., 245, 247
Heinicke, A. J., 134, 136, 147, 148, 279
Heintze, S. G., 92
Heinze, P. M., 454
Hejnowicz, Z., 329, 417
Helgeson, E. A., 381
Helms, K., 354
Helson, V. A., 303
Hemberg, T., 347, 351, 354, 355, 356, 357
Hemming, H. G., 369, 380, 385, 386, 398, 405
Henbest, H. B., 346, 355
Henckel, P. A., 423
Henderson, J. H. M., 347
Hendricks, R. H., 341, 435
Hendricks, S. B., 94, 238, 293, 294, 296, 297, 298, 302, 303, 304, 306, 307, 318
Hendrickson, A. H., 283, 284
Henglein, F. A., 36, 39
Henning, U., 221, 223, 224, 226, 227, 259
Henning, V., 226
Henrici, M., 133
Heppel, L. A., 196
Hepton, J., 39, 53, 54
Heritage, A. C., 143
Hermans, P. H., 35, 36, 40, 42
Hershey, A. D., 332
Hertz, C. H., 412, 421, 422
Hess, B., 199, 211
Hess, K., 37
Hestrin, S., 41, 42
Hewitt, E. J., 63, 64, 65, 66, 70, 72, 79, 80, 456, 459
Hewitt, W. B., 440
Heydemann, A., 95
Heyn, A. N. J., 51, 52, 53, 278
Hibbard, A. D., 272, 283
Hickman, D. D., 240
Hield, H. Z., 375
Hiesey, W. M., 142
Hietala, P. K., 4, 8
Highkin, H. R., 384
Hildebrandt, A. C., 340
Hill, A. C., 441
Hill, G. R., 134, 139,

148, 279
Hill, R., 21, 22, 78, 83, 163, 177, 178, 180, 182, 185, 214
Hill-Cottingham, D. G., 75
Hillman, W. S., 295, 401, 406
Hilz, H., 208, 222
Hinkson, J. W., 178, 179
Hinsvark, O. N., 346, 355
Hiramatsu, K., 139
Hirata, S., 375
Hird, F. J. R., 209, 215
Hirokawa, R., 116
Hirokawa, T., 157
Hirono, Y., 370
Hissink, D. J., 94
Hitchcock, A. E., 441
Hitchcock, D. I., 96, 97
Ho, Hon-Kai, 257
Hoagland, D. R., 93, 99
Hoagland, M. B., 113, 119, 121, 123
Hobbs, M. O., 178
Hoch, F. L., 81
Hoch, G., 155-94; 168, 172, 175, 186
Hock, F. L., 214
Hodge, A. J., 14, 15
Hodges, J. M., 20
Hodgson, J. F., 94, 95
Hoffman, M. B., 148, 279
Hofstee, B. H. J., 95, 103, 104, 105
Hogetsu, K., 143
Holden, F. R., 431
Holdheide, W., 141
Holkova, L., 347, 349, 354, 356, 358, 363
Holley, R. W., 119, 120
Hollingworth, B. R., 126
Hollocher, T., 22,
Holman, R. T., 27
Holmes, R. S., 75, 76
Holmes, R. W., 141, 145
Holm-Hansen, O., 66
Holmsen, T. W., 428
Holmström, B., 162
Holt, A. S., 166
Honda, S. I., 203
Honkanen, E., 4
Hood, D. W., 135, 140
Hood, K. J., 383, 384
Hoop, H., 66
Hoover, C. D., 92
Hooymans, J. J. M., 99, 100, 101
Hope, A. B., 251
Hopkins, H. T., 95, 101, 102, 106
Hopkins, J. W., 128
Horecker, B. L., 210, 460
Hori, Y., 386
Horio, T., 70
Horne, R. W., 36, 44, 58
Horning, M. G., 223
Horreus de Haas, G. H., 168
Horsfall, J. G., 462
Hotta, Y., 121
Houff, W. H., 346, 355
Housley, S., 345, 346, 350, 351, 354, 355, 357, 361
Houwink, A. L., 45, 46, 48
Howard, A., 328, 330
Howard, F. D., 251
Howell, R. W., 372, 378
Hsu, P. H., 93
Huang, R. C., 249, 254
Huber, B., 134, 135, 140, 142
Hubscher, G., 22, 26
Huelin, F. E., 259
Huff, J. W., 221, 222
Huffaker, R. C., 77, 102
Hughes, W. L., 328
Huiskamp, W. J., 168, 169
Hulburt, E. M., 145
Hull, H. M., 432, 434, 435, 439, 440
Hull, J., Jr., 374, 383
Hullin, R. P., 221
Hulme, A. C., 20, 247, 248, 256, 257
Hülsmann, W. C., 117, 120, 123, 250
Humphreys, T. E., 80, 83, 256
Humphries, E. C., 378, 400
Hunnekins, F. M., 105
Hunter, A. S., 282
Hunter, F. E., Jr., 17, 19, 20
Hurwitz, A., 17
Husain, A., 454, 457
Hussey, G., 306
Husson, C., 226
Hutcheson, T. B., Jr., 92
Hutchinson, G. E., 139
Hutschneker, K., 356
Huxley, H. E., 126
Hygen, G., 272, 273, 275
Hylmo, B., 99, 107

I

Icha, F., 347, 349, 354, 356, 358, 363
Ichimura, S., 176
Ichioka, P. S., 64
Ihnen, E. D., 178, 179
Iida, C., 67
Iida, K., 66, 69
Ikeda, K., 299, 301, 302, 303, 305
Ikeda, S., 178, 179, 187
Ikuma, H., 380
Il'ina, A. L., 282
Iljin, W. S., 268, 272, 277
Imamura, S., 295, 310, 311, 312, 316, 317, 318, 370
Imazu, T., 382
Impey, R. L., 75
Ingestad, T., 349
Inglis, S. D., 54
Inkson, R. H. E., 76, 246
Inoue, S., 339
Irvine, J. E., 380
Isherwood, F. A., 37, 58, 82
Ishii, T., 380
Isler, O., 219, 221
Itagaki, E., 67
Itakura, T., 376
Ito, S., 14
Ivanov, V. P., 281
Iwaki, H., 146, 149
Iwakiri, B. T., 374
Iwamura, T., 147
Iyama, J., 148
Izawa, S., 157

J

Jablonski, J. R., 403
Jackman, L. M., 219, 234, 235
Jackson, F. L., 21
Jackson, G. A., 379
Jackson, M. L., 93, 97
Jackson, P. C., 100, 101, 103, 107
Jackson, W. A., 76, 77
Jackson, W. T., 55
Jacob, F., 341
Jacobs, A. L., 208
Jacobs, E. E., 24
Jacobson, L., 100, 102, 106, 337
Jagendorf, A. T., 178, 180, 181, 182, 183, 187, 238
James, T. W., 335
James, W. O., 28, 78, 82, 83, 165, 460
Jamikorn, M., 230, 231, 235
Jamison, V. C., 283
Jang, R., 36, 37, 43, 44, 54, 55, 56, 210, 253
Janick, J., 246, 252
Jännes, L., 8
Jansen, E. F., 36, 37, 54, 55, 56, 210, 253
Jarnefelt, J., 13, 22
Järvinen, H., 6
Jenkin, P. M., 138, 140, 142
Jenkins, W. R., 452
Jenny, H., 97, 107
Jensen, A. E., 136, 138, 142
Jensen, S. L., 229, 234
Jensen, W. A., 40, 58, 337, 401

Jepson, J. B., 350, 356
Jermyn, M. A., 37, 58
Jindra, A., 362
Joel, C. D., 27
Joerrens, G., 300, 307
Joham, H. E., 92
Johansen, R. W., 377
Johnson, B. C., 129
Johnson, C. M., 64
Johnson, D., 18
Johnson, S. P., 382
Johnston, F. B., 255
Johnston, W. H., 270
Joklik, W., 67
Jones, E. R. H., 346, 347, 355
Jones, J. E., 452
Jones, L. G., 280, 282, 286
Jones, L. H. P., 92
Jones, O. T. G., 70, 74
Jordan, D. C., 66
Jordan, W. K., 29
Juday, C., 135, 142, 143
Juday, R. E., 138, 139, 142, 148
Jurinak, J. J., 92

K

Kaben, H., 140, 143
Kägi, J. H. R., 82
Kahn, A., 380, 389
Kahn, J. S., 19, 24, 102
Kahn, J. S., 99, 100, 102, 107, 183
Kaji, J., 461
Kalckar, H. M., 195
Kaldewey, H., 429
Kalf, G. F., 19, 115, 128
Kallio, P., 381
Kalyanasundaram, R., 81, 449, 450, 451, 453, 455, 458, 459, 460, 464
Kamal, M., 454
Kamel, M. S., 137, 145
Kamen, M. D., 165, 166
Kameyama, T., 117
Kamiya, N., 256
Kamprath, E. J., 92, 95
Kandler, O., 196, 207, 215, 239
Kaper, J.M., 345, 346, 361
Kaplan, N. O., 19, 82
Karasek, M., 119
Kardocyssova, E., 141
Kargl, T. E., 230
Karnofsky, D. A., 337
Karnovsky, M. L., 27
Karrer, P., 219
Karström, H., 9
Kasanaga, N., 146, 148, 149
Kasha, M., 187
Kasperbauer, M. J., 372
Kato, S., 378
Kato, Y., 377
Katoh, S., 165
Katoh, T., 157
Katz, E., 186
Katz, J., 256
Kaufman, B. T., 19
Kausch, W., 98, 99
Kautsky, H., 177
Kawarda, A., 371
Keeler, R. F., 64, 65
Keer, T., 268
Kefford, J. F., 257
Kefford, N. P., 347, 350, 353
Keilin, D., 78, 460
Keitt, G. W., 419
Kellenberger, E., 327
Keller, E. B., 116
Keller, P., 107
Keller, S., 308, 309
Kelley, O. J., 282
Kelman, A., 452, 454, 457
Kemp, A. K., 377
Kemp, H. T., 400
Kendrick, J. B., Jr., 432, 434, 435, 440, 443, 444, 445
Kennedy, E. P., 19
Kennell, D., 401
Kennet, B. H., 259
Kenten, R. H., 72, 78, 80
Kenway, C. B., 270
Kenworth, A. L., 279, 280
Kerchow, A., 433
Kern, H., 456
Kernot, B. A., 116
Kerr, T., 35, 38, 51
Kessel, I., 224, 259
Kessler, B., 252
Kessler, E., 81
Kettelapper, H. J., 272
Key, J. L., 18, 251
Keyworth, W. G., 454, 456, 459
Khesin, P. B., 114, 117
Khim, J. X., 196
Khosla, M. C., 219
Khudairi, A. K., 300, 310, 311, 313
Kiermayer, O., 345, 347, 348, 352, 355
Kiese, M., 22
King, H. M., 100, 106
Kingdon, H. S., 119
Kingsley, K., 431
Kinsky, S. C., 79, 80
Kirk, P. L., 334
Kirkland, R. J. A., 204
Kirsch, J. F., 117
Kisliakova, T. E., 138, 142
Kitamura, H., 371
Kittler, M., 208
Kittrick, J. A., 93, 97
Kivilaan, A., 38
Kjaer, A., 134, 141
Klämbt, H. D., 352
Klausmeier, R. E., 7
Klein, A. O., 402
Klein, S., 37, 39
Kleshnin, A. F., 146
Klingman, G. C., 401
Klotz, I. M., 200, 277
Kluepfel, D., 459
Klungsöyr, S., 41
Klute, A., 98
Kmetec, E., 28
Knappe, J., 222
Knauss, H. J., 224
Knight, R. C., 275
Knopka, K., 246, 253, 254
Kobayashi, R., 377
Kobayashi, S., 52, 53
Koch, W., 134
Kogut, M., 67, 74
Koike, M., 23
Kok, B., 155-94; 136, 137, 156, 157, 161, 165, 167, 168, 170, 172, 174, 175, 186
Koller, D., 384
Konar, R. N., 402
Koningsberger, V. V., 119
Könitz, W., 299, 300, 301, 307, 308
Konovalov, Yu. B., 281, 282
Koorneef, P., 378
Kornberg, A., 196, 207, 337
Kornberg, H. L., 207
Korner, A., 117
Kortiz, H. G., 436
Kosan, J. H. D., 341
Koshland, D. E., Jr., 113
Kostychev, S. P., 141, 142
Kozlowski, T. T., 133, 280, 281
Krall, A. R., 181
Kramer, P. J., 99, 107, 133, 135, 141, 148, 205, 267, 268, 269, 271, 277, 278, 279, 280, 421
Krasnovsky, A. H., 155, 167, 178
Krasovskaya-Antropova, T. A., 235
Kraus, J. F., 377
Kretovich, V. L., 2, 360
Kribben, F. J., 295, 306
Krimbas, B. D., 373
Krimsky, I., 207
Krippahl, G., 156, 185
Krishnaswamy, P. R., 119
Kritsman, M. G., 7
Krogmann, D. W., 180, 181, 184, 199
Krone, W., 121
Kröplen-Rueff, L., 221
Krotkov, G., 134, 251, 255
Krzysch, G., 140
Kubo, H., 66, 78
Kuehl, F. A., 129
Kuhn, W., 245, 247

Kuiper, P. J. C., 269
Kulajewa, O., 402
Kulescha, Z., 347
Kumagai, T., 385
Kumazawa, K., 107
Kumm, J., 180
Kummerow, J., 401
Kumisawa, R., 186, 240
Kunitake, G. M., 202, 316
Kunttu, H., 8
Kuntz, J. E., 456, 457, 458
Kupke, G., 358
Kuraishi, S., 398, 400, 401, 404
Kurland, C. G., 126
Kursanov, A. L., 37, 141
Kurtz, L. T., 92
Kurtz, T., 95
Kurz, L., 401
Kusumoto, T., 144
Kutáček, M., 347, 349, 354, 355, 356, 358, 361, 362, 363
Kutsuna, K., 92
Kylin, A., 99, 107

L

Laboureur, P., 386
Lagerwerff, J. V., 93, 94, 99, 103
Laine, T., 6, 7, 359
Lakiza, R. I., 282
Lakshmanan, M., 461
Lakshminarayanan, K., 449, 454, 460, 461
Laloraya, M. M., 402
Lam, S. L., 319
Lamb, R. C., 375
Lamfrom, H., 115, 116, 125
Lambert, J. W., 372, 378
Lamport, D. T. A., 253
Lance, A., 156
Land, D. G., 229, 234
Lang, A., 250, 252, 293, 299, 306, 309, 310, 312, 313, 316, 318, 320, 383, 387, 402
Lansing, A. I., 245, 246, 247
Lantrip, L. W., 149
Lardy, H., 211
Lardy, H. A., 26
Large, C., 378
Larsen, P., 135, 142, 146, 148, 149, 345, 346, 355, 356, 359, 361, 409, 410, 411, 413, 419, 422, 427
Larsen, R. P., 374
Larson, P. R., 377
Lascelles, J., 69
Laties, G. G., 20, 99, 196, 199, 202, 209, 210, 212, 247, 251, 257
Laurencot, J., Jr., 256
Laurie, A., 295
Lavee, S., 373
Laverick, M. L., 144
Lavorel, J., 170
Lawrence, D. B., 136
Lawton, K., 92
Leahy, J., 123, 125
Leak, W. B., 377
Lebedev, G. V., 276
Leben, C., 371, 372
Lebour, M. V., 147
Ledbetter, M. C., 441
Lederberg, E. M., 341
Ledoux, L., 337
Leech, J. H., 38, 39, 48 251, 255, 256, 340
Leech, R. M., 83, 165
Leedale, G. F., 336
Lees, H., 74
Lefort, M., 156
Legg, J. O., 92
Leggett, J. E., 94, 100, 101, 102, 103, 106, 107
Lehman, I. R., 25
Lehninger, A. L., 14, 17, 18, 24, 25, 26, 183, 196, 250
Leinweber, F. J., 309
Leloir, L. F., 40, 44, 195
Lemon, E. R., 279
Leone, I. A., 441
Leopold, A. C., 246, 252, 311, 313, 316, 319, 347, 350, 398
Lesaint, M., 94
Lester, R. L., 13, 19, 25, 27
Letey, J., 98, 283
Levin, A. F., 66
Levine, R. P., 186
Levisohn, I., 379
Levitt, J., 19, 277, 278
Levring, T., 143
Levy, J. F., 17
Lewis, F. J., 272, 274
Lewis, L. N., 374, 383
Lexander, K., 345, 350, 353
Libbert, E., 355, 356, 363
Lieberman, M., 21, 250, 255, 258
Lightbown, J. W., 67, 74
Lijinsky, W., 224, 231
Lima-de-Faria, A., 331
Linck, A. J., 377
Lincoln, R. E., 228, 230, 236
Lincoln, R. G., 312, 318, 319
Lindberg, O., 18, 62, 196, 213
Lindblad, K., 19, 28
Lindop, P., 247
Lindquist, L., 162
Lindsay, W. L., 93
Lindstrom, R. S., 376, 389
Lineweaver, H., 103
Lingrel, J. B., 114, 115, 116, 123, 125
Link, G. K. K., 347
Linkola, H., 9, 10, 66
Linn, B. O., 222
Linnane, A. W., 14, 15, 25
Linser, H., 345, 347, 352, 355, 359
Linshitz, H., 162
Linskens, H. F., 460
Lipetz, J., 348
Lipmann, F., 118, 119, 120, 121, 175, 182, 184, 197, 208, 214
Lippert, L. F., 382, 389
Lipsett, M. N., 17, 18
Littlefield, J. W., 116
Litvinenko, S. N., 381
Liverman, J. L., 293, 295, 299, 306, 310, 311, 312, 313, 317, 346, 355, 361, 362, 397, 406
Livingston, B. E., 274, 275
Livingston, G. A., 345
Livingston, R., 160, 162, 187
Lizandr, A. A., 281, 282
Lloyd, F. E., 268
Lloyd-Jones, C. P., 75
Lockhart, J. A., 252, 293, 295, 302, 309, 310, 311, 316, 317, 370
Loeffler, W., 459
Loewus, F. A., 43, 44
Löhr, E., 143, 144
Lona, F., 293, 317, 318, 380, 384, 385, 389, 399, 406
Long, E. M., 312
Longman, K. A., 313
Loomis, W. E., 135, 138, 139, 140, 267, 277, 279, 295
Loos, G. M., 215
Lopez-Gonzales, J. de D., 97, 98, 107
Lörcher, L., 308
Lorenz, A., 134, 139, 141, 142
Lorenz, O. A., 375
Losada, M., 66, 186
Loughman, B. C., 196
Loustalot, A. J., 273, 282
Low, H., 18, 26
Lowe, D., 369, 385, 386
Lower, H. F., 66
Lownsbery, B. F., 452
Lubell, A., 124
Lucas, G. B., 452
Luckwill, L. C., 346, 353, 354, 374, 375, 386
Ludwig, R. A., 450, 458
Luippold, H. J., 338, 397,

400, 404
Lukton, A., 221, 222, 225
Lumry, R., 185, 187
Lund, H. A., 119, 251, 361, 371
Lund, J.W. G., 134
Lundeen, A. J., 4
Lundegårdh, H., 21, 78, 133, 138, 139, 140, 143, 144, 146, 165, 412
Lunt, O. R., 75
Lusty, C. J., 16
Luzzati, M., 22
Lynch, V. H., 25, 160, 166, 238
Lynen, F., 221, 222, 223, 224, 226, 227, 259

M

Mabey, G. L., 72, 79, 81
McArdle, F. J., 381
McAuliffe, C. D., 94
McCaleb, S. B., 92
McCloskey, P., 369
McCollum, S. P., 230
McComb, A. J., 383
McCorquodale, D. J., 118
McCoy, T. A., 359
McCready, C. C., 64
McCready, R. M., 43
McCune, S. B., 373, 377
MacDonald, I. R., 246, 256, 257
McDonnell, K., 457
MacDougal, D. T., 246
McElroy, W. D., 64, 68, 78, 79, 80
McEwen, H. B., 94
McGivern, M. J., 28
McGregor, S. M., 356, 357
McGregor, W., 135, 141
Machado, R. D., 253, 340
Machlis, L., 36, 58
Maciejewska-Potapczyk, W., 252, 402
McIlrath, W. J., 83, 310, 319
Mackinney, G., 221, 222, 225, 236
McKinney, H. H., 295
Mackler, B., 16, 83
Maclachlan, J. A., 80
McLaren, A. D., 337
McLean, E. O., 107
McLean, J. R., 128
McLeod, G. C., 171, 173, 174, 176, 177
McManus, M. A., 400
McMaster, R. D., 331, 335
McMaster-Kaye, R., 332
McMillan, G. L., 142
MacMillan, J., 369, 370
McMurray, W. C., 26
McNall, E. G., 71
McQuistan, M., 230
McVey, G. R., 376, 385
Mader, P. P., 432
Maeda, E., 419, 427
Maevskaya, A. N., 235
Magill, P. L., 431
Magistad, O. C., 284
Magness, J. R., 280, 283
Mahadevan, S., 355, 356, 361
Mahler, H. R., 19, 66
Majumdar, G. P., 46, 47
Maksimovich, A. E., 277, 281
Malakar, M. C., 347
Maley, G. F., 18
Malini, S., 461
Malison, R., 19, 20
Mallette, F. S., 431
Maloney, M., 94
Malstrom, B. G., 82
Mandel, P., 254
Mandelstam, J., 250
Mann, J., 36
Mann, L. K., 316
Mann, P. J. G., 72, 78, 361
Mann, W., Jr., 43
Manning, W. M., 138, 139, 142, 148, 237
Mapes, M. O., 340
Mapson, L. W., 44, 82, 202, 203
Marcus, A., 250
Mareus, A., 207
Margoshes, M., 65
Margulies, M. M., 180, 181, 182
Marinetti, G. V., 22, 27
Marinos, N. G., 253
Markham, R., 122
Markovitz, A., 40
Marks, J. D., 247, 249, 254
Marrè, E., 195-216; 197, 198, 199, 201, 202, 203, 205, 209, 210, 212, 213
Marshall, S. M., 133, 138
Marsman, J. W., 170
Marth, P. C., 345, 376, 378
Martin, A. R., 37
Martin, E. M., 15, 28, 78
Martin, J. C., 93
Martin, R. P., 100, 196
Martin, W. J., 452
Martinez, L. M., 81
Martin-Smith, C. A., 46, 47, 48
Martius, C., 27
Maruta, H., 148
Marx, W., 20
Maschek, F., 345, 347, 359
Massey, V., 23, 85
Mastalerz, J. W., 376
Mathews, B. C., 94
Mathries, J. C., 81
Matikkala, E. J., 8
Matthaei, G. L. C., 133, 134
Maurer, K., 5
Maximov, N. A., 272, 273, 275, 279, 285
Mayer, A., 136
Mayer, A. M., 255, 388, 400, 402, 406
Mayhugh, M. S., 98
Mayne, B. C., 155, 171, 181, 185
Mayr, H., 345, 347, 359
Mazelis, M., 80, 204
Mazia, D., 339
Mears, K., 340, 397, 403
Medina, A., 70, 71, 80, 81
Medvedev, Zh. A., 113
Mees, G. C., 371
Mehta, R., 129
Meidner, H., 273
Meigh, D. F., 258, 259
Meijer, G., 293, 294, 301
Meiklejohn, J., 73
Meiss, A. N., 77
Meister, A., 7, 119, 359
Melchers, G., 293, 310
Melchior, G. H., 345, 347
Melsted, S. W., 94
Mencl, I., 196
Menke, W., 156
Menten, M. L., 101
Menzel, D. W., 144
Menzel, R. G., 94, 106
Mercer, F., 155, 251, 253, 255, 256
Merrill, M. H., 446
Mersereau, J. D., 436
Mes, M. G., 282, 379, 381
Meselson, M., 332
Metzner, P., 428
Meudt, W., 350, 353, 354
Meyer, B. S., 141, 143, 268
Meyer, K. H., 35
Michaelis, L., 101
Michailidis, E., 373
Michel, B. E., 357
Middleton, J. T., 431-48; 144, 431, 432, 433, 434, 435, 436, 437, 440, 441, 443, 444, 445, 446
Miettinen, J. K., 7, 8, 9, 10
Miller, C. O., 395-407; 396, 397, 398, 399, 400, 403, 405, 406
Miller, F. M., 347
Miller, G. W., 23, 76
Millerd, A., 250
Millington, W. F., 319
Mills, G. T., 40
Milner, H. W., 142, 269, 280
Milthorpe, F. L., 267, 270, 272, 273, 275
Minakami, S., 19
Mirsky, A. E., 128
Misch, L., 35
Misra, P., 37
Mitchell, H. K., 78

Mitchell, J. W., 345, 376, 378
Mitchell, M. B., 78
Mitchell, R. L., 63, 66
Mitra, G. C., 399, 400
Mitra, P., 347, 356, 358
Mitra, S., 331
Mitsui, E., 381, 384
Mitsui, H., 5
Mitsui, S., 107
Mituya, A., 147
Miyachi, S., 157, 158
Modi, V. V., 236
Moewus, F., 401
Moffatt, J. S., 369, 370, 371
Mohr, H., 428
Moldave, K., 119, 123
Molisch, H., 246
Mollenhauer, H. H., 15, 38, 39, 48, 251, 255, 256, 340
Möller, C. M., 136, 146
Mönch, I., 140
Monod, J., 210
Monselise, S. P., 252
Monsi, M., 143, 145, 146, 148, 149
Montavon, M., 219
Montermoso, J. C., 269, 271
Montfort, C., 143
Moore, A. T., 37, 45, 46
Moore, C. W., 376
Moore, K. B., 25
Moore, T. C., 382
Moraw, R., 81, 162, 164
Moreland, D. E., 401
Morgan, D. G., 371, 420
Morgan, J. F., 93
Morimura, Y., 336
Morris, A., 123, 125
Morrison, M., 22
Morrison, R. I., 76
Mortensen, L. E., 66
Mortenson, L. E., 7
Mortland, M. M., 92, 94, 95
Morton, R. K., 15, 22, 28, 67, 78, 195, 200, 204
Möslein, E. M., 223, 226, 227
Moss, D., 279
Mothes, K., 250, 281, 402
Mott, G. O., 279
Mower, H. F., 7
Mudd, J. B., 359
Mudd, S. H., 207
Mudrack, F., 134
Mueller, R. T., 75
Mühlethaler, K., 46
Muir, C., 246
Muir, R. M., 345
Muir, W. H., 340
Mulholland, T. P. C., 369
Mullaney, P. D., 373
Müller, A., 162, 164, 169
Müller, D., 135, 136, 137, 138, 141, 142, 144, 145
Müller, R., 345
Munekata, H., 378
Munro, H. N., 117
Murata, Y., 148
Murneeck, A. E., 197, 198, 293, 345, 346
Murphey, W. K., 376
Murray, R., 22
Musaeva, L. D., 281
Musgrave, R. B., 279
Myers, J., 137, 171, 172, 173, 174
Mysina, A. V., 281
Myszka, A., 84

N

Naef-Roth, S., 458, 459, 461
Nagao, M., 381, 384, 385
Nagy, E., 235
Najjar, V. A., 70, 71
Nakamoto, T., 181
Nakamura, H., 178, 180
Nakamura, K., 5, 346, 356, 361
Nakamura, Y., 37
Nakayama, S., 301, 302, 303, 306, 312, 314
Nakayama, T. O. M., 221, 225, 229, 234, 236
Nance, J. F., 55
Nanda, K. K., 307, 308, 310, 363
Naori, H(atsudo), 333
Naori, H(iroto), 333
Nasatir, M., 334
Nash, A. S., 373
Nason, A., 25, 64, 65, 67, 71, 72, 74, 83
Nasr, T., 313
Nathans, D., 117, 123
Naylor, A. W., 203, 293
Naylor, F. L., 319
Naylor, J., 396
Naylor, J. M., 381
Neal, D. C., 452
Neal, G. E., 20, 248, 256
Neary, G. J., 246
Neatby, K. W., 270
Needham, J., 265
Neely, P. M., 371
Negisi, K., 279
Neish, A. C., 40, 43, 44, 55, 205
Nekrassova, T. V., 386
Nelson, J. L., 94
Nelson, L. B., 98
Nelson, N., 431
Nelson, P. M., 379
Nelson, T. C., 376
Nelson, W. L., 95
Němec, B., 414
Nestel, L., 251
Neufeld, E. F., 40, 41, 43, 44, 195
Neuhaus, F. C., 118
Neuman, J., 357
Neumann, G., 381, 400, 406
Neumann, N. P., 22
Neuwohner, W., 142
Newcomb, E. H., 28, 38, 48, 54
Newhall, A. G., 452
Newson, L. D., 452
Newton, A. A., 335
Newton, J. W., 15
Nezgovorova, L. A., 277, 280
Ng, E. K., 54, 356
Nichiporovich, A. A., 144, 145, 146, 277
Nicholas, D. J. D., 63-90; 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 74, 77, 79, 80, 81
Nichols, R., 350, 354
Nicolas, R., 22
Niedergang-Kamien, E., 246, 252
Niederpruem, D. J., 18, 20, 21, 209, 214
Nielsen, J., 136
Nielsen, N., 378
Nielsen, T. R., 106
Nieman, R. H., 99, 178, 180, 378
Nihei, T., 147
Nishimura, M., 165, 189
Nishimura, S., 458
Nisman, B., 118
Nitsch, C., 350
Nitsch, J. P., 311, 313, 347, 350, 361, 376, 382
Noble, W., 432, 434, 439, 440
Noble, W. M., 435
Nobs, M. A., 142
Noggle, J. C., 95, 99, 100, 101, 102, 103, 106
Nomoto, N., 135, 141
Norgard, D. W., 221, 222, 229, 231
Norland, M. A., 379
Noro, T., 375
Norris, K. H., 258, 296, 297
Northcote, D. H., 36, 39
Norton, A. G., 251
Novelli, G. D., 117, 118, 119, 207
Novikoff, A. B., 15, 28
Novitskiy, Y. I., 277
Novoderzhkina, Yu. G., 84
Nozaki, M., 66, 186
Nutman, F. J., 141

O

Oaks, A., 80, 256
Oberdorfer, E., 143
Oberländer, H. E., 95, 100, 107
Ochoa, S., 114, 129
Odhnoff, C., 83
Odum, H., 135, 140
Ofengand, E. J., 119, 120, 121
Ogata, K., 116
Ogawa, Y., 295, 312, 370
Oguri, M., 141
Oh-Hama, T., 157, 158
Ohle, W., 141
Ohmachi, K., 66, 69
Okada, T. A., 337
Okamoto, T., 107
Okauenko, A. S., 277, 281
Okumura, F. S., 397, 398, 400
Okunuki, K., 23, 70
Olivo, C. D., 356
Olmstead, A. J., 257
Olsen, R. A., 93, 94
Olsen, S. R., 92, 95
Olson, J. M., 168, 189
Olson, M. E., 116
Olson, R. A., 155, 163, 174
Omori, S., 116
Oohata, T., 374
Oppenheimer, H. R., 272
Ordin, L., 44, 47, 55, 56, 57, 210, 274, 278, 280
Orii, Y., 23
Orr, A. P., 133, 138
Osada, A., 148
Osawa, S., 121, 122, 128
Osawa, T., 382
Osborne, D. J., 250, 252, 253, 357
Osborne, L. D., 383
Osman, H. G., 229, 234
Osterhout, W. J. V., 100
Ota, A., 70
Otaka, E., 121, 122
Overbeck, F., 417
Overbeek, J. T. G., 119
Overholser, E. L., 279, 280
Overstreet, R., 100, 102, 106, 107
Ovington, J. D., 136
Owen, J. H., 456
Owen, P. C., 277, 282

P

Pack, M. R., 441
Packer, L., 14, 16, 17, 18, 19
Padilla, G. M., 335
Page, O. T., 449, 456, 458
Painter, R. B., 328
Pal, J. C., 347, 357
Palache, C., 92
Palade, G. E., 14, 15, 117, 126, 156
Paleg, L. G., 379
Palmcrantz, P. J., 251
Palmer, J. H., 415
Palmer, L., 432
Palmer, M. G., 77
Palser, B. I., 83
Pánczél, M., 235
Paquin, R., 377, 460
Park, J. K., 372, 378
Park, K., 135, 140
Parker, F. W., 93
Parker, M. W., 293, 294, 296, 298, 303, 308
Parmeter, J. R., 456, 457
Parups, E., 373
Pashkar, S. I., 347, 352
Patau, K., 338, 397
Pate, J. S., 345, 347, 353
Patwa, D. K., 236
Paulauskaite, K. P., 114
Paulus, A. O., 435, 443, 445
Pauly, H., 14, 16, 19
Pavillard, J., 348, 349
Pearsall, W. H., 136
Pearse, H. L., 272, 273, 275
Pearson, G. A., 97
Pearson, J. A., 250
Pearson, L. C., 136
Peech, M., 93, 94, 99
Pegg, G. F., 347, 348, 451, 456, 461, 464
Pejve, J. V., 84
Pelc, S. R., 328, 330
Pelter, A., 223
Penefsky, H., 25
Penn, N. W., 248
Pennell, G. A., 209
Penner, J., 312
Perlin, A. S., 56
Perlis, I. B., 55
Person, C., 250, 252
Perry, R. P., 336
Peters, D. B., 283, 285
Peters, T., Jr., 116
Peterson, C. E., 379, 389
Peterson, D. F., 373
Peterson, M. L., 279, 280, 281, 282, 286, 295
Petinov, N. S., 277, 281
Petracek, F. J., 236
Petrack, B., 175, 182, 184
Petraschkaite, S. K., 114
Petrie, A. H. K., 281
Petro, H. B., 270
Petrushka, E., 24
Pettersson, S., 107
Petty, J. H. P., 252, 382, 383
Phagpolngarm, S., 235
Phan, Chon-Ton, 257
Philip, J. R., 267, 271, 283
Philips, I. D. J., 348, 349, 354
Phillips, A. H., 224, 259
Phinney, B. O., 312, 369, 371, 380, 389
Pichler, J., 428
Pierpoint, W. S., 20, 24, 26
Pierre, W. H., 93
Pierson, C. F., 457
Pieters, G. A., 144
Piiroinen, P., 381
Pilet, P. E., 345, 349, 350, 353, 360, 428
Pinchot, G. B., 20, 27
Pioth, L. C., 348
Pirie, N. W., 250
Piringer, A. A., 303
Pisek, A., 134, 135, 138, 139, 140, 141, 143, 144
Pittendrigh, C. S., 304, 305, 307, 308, 309
Plaisted, P. H., 257
Platt, R. S., 350, 401
Pleshkov, B. P., 347, 349
Pletscher, A., 221
Plummer, T. H., 398
Polis, B. D., 250
Poljakoff-Mayber, A., 351, 381, 400, 406
Pollard, C. J., 25
Pollard, J. K., 257
Pollock, E. G., 401
Pollock, J. R., 378
Polster, H., 138, 141, 142, 148
Polyanovskii, O. L., 360
Popham, R. A., 319
Popják, G., 220, 223, 227
Popovskaya, E. M., 281
Porter, J. W., 221, 222, 224, 228, 229, 230, 231, 236
Porter, K. R., 253, 340
Possingham, J. V., 79
Post, K., 385
Post, L. C., 345, 346, 354, 355, 362
Potapov, A. A., 143
Powell, L. E., 346, 375
Powell, R. D., 398, 406
Prat, H., 266, 268
Pratt, H. K., 257
Preiss, J., 119, 120, 121
Prescott, D. M., 246, 332
Preston, R. D., 35, 36, 39, 45, 46, 47, 48, 50, 52, 53, 54, 274
Prialgauskaite, L. L., 235
Price, C., 316
Price, C. A., 16
Priestly, J. H., 272
Primer, P. E., 374
Printz, H., 143

Procházka, Z., 347, 349, 354, 356, 358, 359, 361, 362, 363
Prosser, M. V., 379
Provasoli, L., 399
Prowse, G. A., 139, 142
Pruskova, L. D., 281, 282
Pullman, M. E., 25
Pullmann, A., 195, 200
Pullmann, B., 195, 200
Purcell, A. E., 222, 229
Purves, W. K., 38, 48, 51, 345, 356, 357
Purvis, E. R., 77
Purvis, O. N., 384

Q

Quackenbush, F. W., 230
Quastel, J. H., 1, 24
Quastler, H., 328

R

Raacke, I. D., 114, 115, 128, 255
Raade, R. D., 455
Raadts, E., 354, 362
Raaflaub, J., 16
Rabinovitz, M., 116
Rabinowitch, E. I., 133, 136, 137, 138, 141, 162, 165, 166, 168, 169, 170, 171, 172, 173, 174, 176, 185, 187
Rabinowitz, J. C., 196
Rabinowitz, J. L., 221
Rabson, R., 257
Racker, E., 25, 199, 211, 214
Racusen, D. W., 250
Radcliffe, J. C., 431
Radley, M., 370, 371
Rafos, S., 371
Ramirez, J., 165, 166, 189, 240
Ranby, B. G., 35
Raney, F. C., 265-92
Ranson, S. L., 20, 76
Rappaport, D. A., 196
Rappaport, L., 382, 383, 384, 389
Rasch, E., 330, 331
Rautanen, N., 3, 5
Raven, K. A., 318, 319
Rawitscher, F., 415
Ray D., 282
Ray, P. M., 36, 37, 39, 345
Raya, M., 272
Raymond, J. C., 379
Redemann, C. T., 346
Redfearn, E. R., 226, 235
Redmond, W. J., 66
Reed, G. B., 251
Reed, L. J., 23
Reemberg, B., 81
Reeve, R. M., 257
Regeimbal, L. O., 280
Rehner, G., 139
Reiff, B., 428
Reinert, J., 359, 398
Reingard, T. A., 347, 352
Reis, P. J., 19, 28
Reisener, H.-J., 423
Reiske, J. S., 185, 187
Reiss, E., 422
Reitemeier, R. F., 93, 94
Remmert, L. F., 24, 26, 250
Rendi, R., 128, 253
Renner, O., 269
Renz, J., 459
Resende, F., 295
Retovsky, R., 355
Reusser, P., 461
Revel, J. P., 14
Revell, S. H., 337
Rhoads, W. A., 76, 77
Rice, B., 20
Rich, M. A., 337
Rich, S., 445
Richards, B. L., 440
Richards, L. A., 98, 99, 282, 283, 284
Richardson, S. D., 141, 144, 380
Richmond, A. E., 250, 252, 402
Richmond, P. T., 252, 382, 383
Richter, G., 332
Rickes, E. L., 129
Riggio-Bevilacqua, L., 199
Riker, A. J., 340, 456, 457, 458
Riley, G. A., 147
Ringelmann, E., 222
Ripley, G. W., 48
Ris, H., 14, 15
Rishbeth, J., 454
Roach, J., 320, 387
Röbbelen, G., 188
Robbins, W. R., 76, 77
Roberts, A., 94
Roberts, D., 328
Roberts, E., 337
Roberts, E. H., 350, 419
Roberts, E. R., 66
Roberts, R. B., 125, 127
Roberts, R. H., 312
Robertson, R. N., 99, 209, 250, 251
Robins, J. S., 282
Robinson, F. M., 222
Robinson, W., 251
Rodhe, W., 136
Rodrigo, F. A., 169
Rodrigues, G., 92
Roelofsen, P. A., 35, 36, 37, 38, 42, 44, 45, 46, 47, 48, 49, 50, 52
Rogers, B. J., 250
Roine, P., 3
Rokach, A., 268
Rokosova, K., 355
Rollins, M. L., 37, 45, 46
Romney, E. M., 77, 80
Romose, V., 135, 139, 146, 148
Ronney, C. D., 379
Roosen, P., 268
Roosheroe, I., 410
Rosen, W. G., 81
Rosenberg, L. L., 201
Rosenberg, T., 100
Rossi, G., 202, 203
Rossman, E. C., 379
Rotblat, J., 246
Rothbuhr, L., 174
Rothrock, J. W., 376
Rouiller, Ch., 251, 253
Roussos, G. G., 71, 72
Roux, E., 226
Rowan, K. S., 197, 198, 199, 204, 213
Rubins, E. J., 93, 94
Rubner, M., 246
Rudney, H., 221, 223
Rudzinska, M. A., 246, 253, 254
Ruegg, R., 219, 221
Rufelt, H., 409-30; 349, 410, 411, 413, 416, 417, 418, 427, 428
Rumberg, B., 162
Runeckles, V. C., 134
Russell, F. S., 147
Russell, M. B., 265, 266, 267, 270, 280, 283, 285, 286
Russell, R. S., 100, 107, 283
Ruttner, F., 133, 137, 142, 143
Ryther, J. H., 138, 139, 141, 145, 147

S

Sacher, J. A., 246, 252, 253
Sachs, J., 134
Sachs, R., 318
Sachs, R. M., 295, 312, 320, 387
Sadana, J. C., 68
Sadasivan, T. S., 449-68; 458, 460, 464
Saeki, T., 135, 141, 143, 145, 146, 148, 149
Sager, R., 156, 161, 169, 237
Saini, A. D., 282
Saiz del Rio, J. F., 94
Salisbury, F. B., 293-326; 293, 295, 298, 299, 300, 306, 308, 309, 310, 311, 312, 313, 314, 315, 316,

317, 318, 319
Salkowski, E., 350
Saltman, P., 202, 316
Samborski, D. J., 250, 252, 256
Sanadi, D. R., 24
Sanchez, C., 92
Sanchez Nieva, F., 359, 361
Šanda, V., 347, 354, 356, 358, 359, 362, 363
Sandegren, E., 378, 389
Sander, G., 396
Sando, W. J., 295
Sandoval, J. A., 312
Sands, R. H., 23, 27, 83
Sannella, J. L., 35, 36
San Pietro, A., 180, 185
Santa Maria, R., 267
Sanwal, B.D., 450, 453, 456, 461
Saperstein, S., 230
Sapozhnikov, D. I. I., 235
Saraswathi-Devi, L., 81, 458, 460, 464
Sargent, M. C., 149
Saris, N. -E., 4, 6
Sarkanen, K., 162
Sarojini, T. S., 460
Sasa, T., 147
Sasser, J. N., 452
Satchell, D., 98
Sato, C. S., 44
Sato, C. W., 19
Sato, E. S., 207
Sato, R., 67, 72
Sato, S., 340
Satto, T., 279
Satyanarayana, G., 454, 459, 461
Sauberer, F., 133, 136, 142, 143
Saucy, G., 221
Saz, A. R., 81
Scarborough, H., 347, 357
Scarisbrick, R., 22, 78
Scawin, J. H., 79
Schachtschabel, D., 121
Schaeffer, P., 341
Schanderl, H., 134
Scheffer, R. P., 457, 461
Scherbaum, O. H., 334, 335
Scheuerman, R., 358
Schidlovsky, G., 156
Schilling, G., 92
Schlegel, H. G., 233
Schlessinger, D., 126
Schmid, C., 20
Schmidt, G., 204
Schneider, G. W., 280
Schneider, K. C., 66
Schneider, K. H., 81
Schneider, W. C., 13, 26
Schneiderman, H., 210
Schoch-Bodmer, H., 49
Schocken, V., 356
Schoeneweiss, D. F., 457
Scholefield, P. G., 24
Schöne-Schneiderhöhn, G., 309
Schotz, F., 239
Schramm, M., 41, 42
Schrandolf, H., 398
Schrank, A. R., 401, 420
Schroeder, W. A., 236
Schubert, J., 97
Schuller, P. , 168
Schulte, H. F., 431
Schutz, B., 17, 19, 20
Schwabe, W. W., 299, 310, 316
Schwalm, H. W., 432, 435, 443
Schwan, H. P., 14, 16, 19
Schwarz, K., 25, 345, 346, 361
Schweet, R. S., 115, 116, 119, 120, 123, 125
Schwemmle, B., 312
Scott, A. D., 92
Scott, C. O., 95
Scott, E. G., 83
Scott, F. M., 44, 45, 272
Scott, J. F., 121, 122, 123
Scott, R. A., 397, 406
Scott, W. E., 439, 440, 444, 446
Scully, N. J., 294, 295, 298, 310
Scurfield, G., 371, 376
Seaman, D. E., 198, 213
Seaman, G. R., 114
Seaton, J. C., 369, 370
Seegmiller, C. G., 43
Seeley, R. C., 355, 356, 359, 361
Sei, I., 314
Seigler, W. E., 372, 378
Sekuzu, I., 23
Selim, H. H. A., 317
Sell, H. M., 346, 348, 355, 371, 374, 375, 379, 386, 389
Selman, I. W., 347, 348, 451, 454, 456, 461, 464
Semikhatova, O. A., 142
Sen, S. P., 316, 345, 350, 352
Sen-Gupta, P. N., 347, 357
Sequeira, L., 455
Servettaz, O., 203
Seta, V., 371
Setterfield, G., 35-62; 38, 39, 45, 46, 47, 48, 49, 51, 52, 55, 56, 255
Seybold, A., 143
Shafer, J., Jr., 272
Shafizadeh, F., 40, 46
Shannon, L. M., 75, 77
Shantz, E. M., 345, 350, 351, 397, 403, 405
Shapiro, R. E., 91-112; 93, 94, 95, 96, 98, 99
Sharon, N., 118, 119
Sharrah, J. S., 431
Shatkin, A. J., 155, 156
Shaulis, N., 374
Shaw, E., 94
Shaw, K. N. F., 345, 363
Shaw, M., 256
Shaw, R. H., 282
Shealtiel, M., 99, 209
Shean, J., 19
Shear, G. M., 454
Sheeham, T. J., 420
Shepherd, C. J., 128
Sherman, F. G., 328
Sherwood, H., 187
Shibata, K., 147
Shibuya, T., 428
Shigeura, H. T., 126
Shikata, S., 5
Shimada, B. M., 141
Shimbayashi, K., 253
Shimizu, T., 148
Shimp, N. F., 23, 24
Shiraki, S., 376
Shiraki, Y., 376
Shive, W., 387, 400, 401, 402, 406
Shmukler, H. W., 250
Shneour, E. A., 208, 222, 223, 236, 248
Shorr, E., 196, 197
Shorrocks, V. M., 100, 107
Shreve, E. B., 269
Shug, A., 66
Shulgin, I. A., 146
Shunk, C. H., 222
Siegelman, H.W., 250, 296, 297, 304
Siegesmund, K. S., 48
Siekevitz, P., 14, 16, 117, 126
Silver, W. S., 69
Siminovitch, L., 332
Simkin, J. L., 113, 125, 128
Simon, E. W., 23
Simon, H., 423
Simpson, G. M., 362
Simpson, J. R., 74
Simpson, M. V., 19, 115, 116, 128, 248, 251
Simson, P., 125, 127
Sinex, F. M., 245, 247
Singer, M. F., 121
Singer, T. P., 16, 23, 85
Singh, R. N., 66
Siroli, G. S., 358
Sisken, J. E., 330, 331, 337
Sisler, E. C., 21, 23
Sissins, M. E., 229, 233, 234
Sistrom, W. R., 161, 239
Sjostrand, F. S., 14, 15
Skeggs, H. R., 222
Skinner, C. G., 387, 399, 400, 401, 402, 404, 406
Skok, J., 83, 84, 310
Skoog, F., 37, 55, 66, 278,

338, 360, 396, 397, 398, 399, 403, 405
Skow, R. K., 163
Slater, E. C., 13, 14, 24, 25, 26, 27, 250
Slater, W. G., 43, 205
Slatyer, R. O., 267, 269, 270, 271, 272, 276, 277, 283, 284
Slavik, B., 271, 275
Sleeth, B., 457
Slie, R. B., 81
Smillie, R. M., 255
Smith, A. L., 452
Smith, C. R., 375
Smith, E. E. B., 40
Smith, G. F., 345, 346, 350, 355, 361
Smith, J., 340
Smith, J. D., 121, 404
Smith, J. H. C., 188
Smith, L., 14, 165, 166, 168, 189, 202, 240
Smith, R. G., 431
Smith, R. L., 75, 107
Snyder, F. W., 383, 384
Snyder, J. L., 36
Snyder, W. C., 454
Sober, H. A., 7
Söding, H., 354, 358, 362, 420
Sokolova, N. A., 281
Sondheimer, E., 357
Soost, R. K., 380
Sorbo, B., 221
Šorm, F., 354, 356, 358, 362, 363
Sorokin, C., 139
Sorokin, H. P., 28
Sorokin, S., 28
Sorokin, Yu. I., 146
Spahr, P. F., 121, 404
Spear, I., 316
Spector, W. S., 295
Speer, R. J., 94
Spencer, D., 69, 72, 78
Spencer, D. M., 249
Spencer, E. J., 270, 272, 273, 276
Spencer, E. L., 189
Spencer, H. G., 272, 273, 275
Spencer, M. S., 26, 247, 248, 250, 257, 258
Spiegelman, S., 114
Spikes, J. D., 155, 171, 185, 187
Spit, B. J., 47
Spoehr, H. A., 269, 280
Spolter, H., 202
Spruit, C. J. P., 160, 174
Spurr, A. R., 83
Srinivasan, B. D., 338
Stadtman, E. R., 259
Stafford, H., 28
Stahlberg, S., 92, 94
Stålfelt, M. G., 133, 134, 137, 138, 139, 141, 148, 269, 272, 275
Stamm, A. J., 273
Stanescu, P. P., 269
Stanier, R. Y., 161, 186, 227, 229, 231, 234, 239, 240
Stannard, J. N., 460
Staples, R. C., 77
Starr, M. P., 230
Stacher, H., 48
Steelink, C., 350
Steemann Nielsen, E., 133, 135, 136, 138, 140, 142, 144, 145, 146, 147
Stefan, J., 272
Stefansson, B. R., 372, 378
Steinberg, D., 248
Steinberg, R. A., 10
Steiner, M., 7
Stephens, E. R., 433, 434, 439, 440, 441, 444, 446
Stephenson, M. L., 120, 121, 122, 123, 251
Sterling, C., 47
Stern, B. K., 185
Stern, H., 255, 334, 335
Stetten, D., Jr., 256
Stevens, H. M., 67, 68
Stevenson, F. J., 92
Steward, F. C., 8, 257, 340, 345, 350, 351, 397, 403, 405
Stewart, C. P., 347, 357
Stewart, G. R., 93
Stewart, W. S., 373
Stich, H. F., 338
Stiles, W., 269
Stinson, R. F., 295
Stitts, C., 202
Stocker, O., 135, 136, 138, 139, 140, 141, 144, 269
Stocking, C. R., 29, 255, 265, 267, 387
Stockton, J. R., 286
Stodola, F. H., 369
Stoedsenius, W., 14
Stoll, A., 133, 135
Stoll, C., 459
Stolwijk, J. A. J., 76, 293, 301, 303
Stone, B. A., 40, 42
Stone, E. C., 270, 295
Stotz, E., 22, 27
Stout, P. R., 64
Stowe, B. B., 345, 346, 347, 350, 351, 355, 356, 361, 369
Stoy, V., 380
Straub, F. B., 117
Street, H. E., 350, 356, 357
Strehler, B. L., 160, 161, 166
Strittmatter, C. F., 22
Strominger, J. L., 44
Strong, F. M., 395, 397, 402, 403, 404, 405
Struckmeyer, B. E., 456, 458
Strugger, S., 274
Stuart, N. W., 369-94; 376, 377, 378, 379, 382, 383, 385, 389
Stulberg, M. P., 119
Stumpf, P. K., 196, 207, 208, 249, 259
Stutz, E., 356
Subba-Rao, N. S., 455, 456
Sublett, T. H., 359
Subramanian, D., 449, 454, 458, 460, 461
Sudia, T. W., 377
Sugino, M., 316
Sulochana, C. B., 453, 460
Sumiki, Y., 371
Sumpter, W. C., 347
Sundman, J., 8
Suomalainen, H., 8
Surrey, K., 176
Sussex, I. M., 356, 357
Sutcliffe, J. F., 209
Suter, P. J., 369, 370
Sutherland, L., 378
Sverdrup, H. V., 147
Swann, M. M., 334
Swift, H., 328, 331
Sykes, J. T., 347
Szalai, I., 347, 349
Szweykowska, A., 400
Szybalski, W., 337

T

Tackett, D. L., 345
Tafel, J., 8
Tagawa, K., 66
Tagawa, T., 39, 53, 54
Tager, J. M., 248, 250, 256
Takahashi, H., 65, 72
Takahashi, N., 371
Takai, M., 371
Takatori, F. H., 375
Takeda, T., 148
Takemori, S., 23
Takimoto, A., 295, 299, 301, 302, 303, 305, 310, 311, 317, 318
Talbert, F. D., 387, 400, 401, 402, 406
Talboys, P. W., 454, 456, 458
Talling, J. F., 133-54; 134, 135, 136, 137, 138, 139, 140, 142, 145, 147, 149
Tamari, K., 461
Tamiya, H., 137, 147, 157, 158
Tamm, E., 140

Tamura, S., 371
Tanaka, T., 385
Tandler, M. D., 66
Taniguchi, S., 5, 66, 67, 69, 72
Tanner, H. A., 81, 158
Tappel, A. L., 18, 25
Tarakanova, G. A., 421
Tarnanen, J., 1
Tashima, Y., 295, 311, 316
Tatham, P., 79
Taussky, M. H., 196, 197
Tavormina, P. A., 221, 222, 223
Taylor, G. S., 441, 445
Taylor, H. F., 355, 359, 361
Taylor, J. H., 327-44; 328, 329, 330, 331, 333, 334, 335, 341
Taylor, O. C., 434, 435, 436, 437, 439, 440, 441, 444, 445, 446
Taylor, S. H., 330
Taylor, W. C., 345, 346, 347, 350, 355, 361
Tchen, T. T., 207, 224
Teale, F. W. J., 170, 238
Teär, J., 350
Teas, H. J., 420, 428
Tedeschi, H., 15
Terrell, A. J., 83
Teubner, F. G., 197, 198, 346
Thalacker, R., 29
Thein, M. M., 320
Thimann, K. V., 37, 51, 52, 53, 54, 56, 76, 82, 134, 203, 205, 210, 215, 248, 251, 254, 257, 259, 345, 346, 347, 350, 351, 355, 356, 357, 361, 380, 399, 402, 405, 406
Thoday, D., 134
Thomas, J., 210
Thomas, J. B., 155, 170, 174, 175, 176, 185, 237
Thomas, M. D., 133, 134, 139, 148, 149, 279, 431, 435
Thompson, G. A., 222, 229
Thompson, J. R., 372, 378
Thompson, L. C., 143
Thompson, P. A., 310, 385
Thorn, M. B., 21
Thorne, D. W., 92
Threlfall, R. J., 456
Thurber, G. A., 379
Thurlow, J., 313
Thut, H. F., 277
Tice, S. V., 7
Tiffin, L. O., 75
Tilson, D., 328
Timell, T. E., 36
Timm, H., 382, 389
Timonin, M. I., 452
Tisdale, H., 22
Tissières, A., 78, 121, 126, 127
Tober, C. W., 15
Tobita, T., 123
Tod, H., 381
Todd, A. R., 122
Todd, G. W., 437, 438, 441, 442, 445
Tognoli, L., 20, 29, 203
Tolbert, N. E., 375, 377, 387, 388, 400, 406
Tolinshis, L. E., 114
Tombesi, L., 281
Tomes, M. L., 230
Toole, V. K., 370, 371, 380, 381, 389
Topps, J. H., 249
Tori, L., 22
Torrey, J. G., 397, 398, 401
Torsell, K., 83
Toschevikova, A. G., 281
Toth, S. J., 80
Towers, G. H. N., 8
Tranquillini, W., 134, 138, 139, 140, 141, 143, 144, 148
Transtrum, L. G., 441
Treharne, R. W., 158
Trelease, S. F., 208
Trenk, B., 245, 247
Treshow, M., 441
Trezzi, F., 358
Tripp, V. W., 37, 45, 46
Trurnit, H. J., 156
Tschenokov, V., 141, 142
Tschudy, R. H., 143
Ts'o, P. O. P., 19, 124, 125, 254
Tso, T. C., 373
Tsui, C., 359
Tsukamoto, Y., 349
Tsuno, Y., 148
Tua, C., 29, 203
Tukey, H. B., 345
Tupy, J., 347, 349, 354, 356, 358, 363
Turchina, V. S., 235
Turk, A., 431
Turner, D. H., 197, 198, 199
Turner, J. F., 199, 204, 212, 213
Turner, J. S., 198, 213
Tuttle, A. L., 240
Tyszkiewicz, E., 94

U

Uehara, G., 92
Uhl, A., 140
Uhler, R. L., 77
Uhring, J., 379
Uksila, E., 8
Ullmann, A., 117
Ulrich, R., 247, 257
Upchurch, R. D., 280, 282, 286
Upchurch, R. P., 279, 280, 281

V

Vaadia, Y., 265-92; 269, 270, 285, 286
Vaccaro, R. F., 145
Vagelos, P. R., 259
Valenta, M., 347, 349, 354, 356, 358, 363
Valle, E., 9
Vallee, B. L., 65, 72, 81, 82
van Andel, O. M., 269
van Bavel, C. H. M., 282
Van den Ende, J., 378
van den Honert, T. H., 99, 100, 101
Van der Hart, J. M., 168, 169
van der Marel, H. W., 92
van der Veen, R., 293, 301
Vander Wende, C., 23, 24
Van De Ven, A. M., 119
Vanecko, S., 249, 254
Van Eys, J., 82
van Iterson, G., Jr., 49
Van Niel, C. B., 186, 233
van Oorschot, J. L. P., 136, 137, 147
van Overbeek, J., 278, 279, 309, 345, 356
van Raalte, M. H., 356, 357
Van't Hof, J., 329, 337
van Ysselstein, M. W. H., 355, 356
Varadarajan, P. D., 460
Vareschi, V., 145
Varga, M. B., 349, 352
Varma, T. N. R., 225
Varner, J. E., 245-64; 4, 64, 65, 73, 247, 248, 249, 254
Varoczy, E., 84
Vasil, I. K., 341, 401
Vasil'eva, N. G., 276
Vatter, A. E., 19, 20, 28, 155, 156, 240, 251
Vaughan, M., 248
Veeger, C., 23
Veihmeyer, F. J., 283, 284
Velasco, J., 250
Veldstra, H., 345, 346, 357, 361
Veldstra, L., 25
Velick, S. F., 22
Vengris, J., 107
Venkata Ram, C. S., 459, 461
Venkataraman, A., 22, 23, 24

Vennesland, B., 80, 178, 180, 185, 199, 202, 207
Venning, F. D., 51
Verduin, J., 135, 138, 139, 140, 142, 149, 272, 279
Verkerk, K., 303
Vernon, L. P., 178
Vickery, H. B., 250
Viets, F. J., Jr., 94, 99
Viglierchio, D. R., 452
Vinberg, G. C., 133, 135
Vinograd, J., 116, 254
Virgin, H., 269
Virtanen, A. I., 1-12; 1, 2, 4, 5, 6, 7, 8, 9, 10, 66, 78, 359
Vittorio, P. V., 251
Vlamis, J., 93, 94
Vlasyuk, P. A., 80
Vlitos, A. J., 358, 359
Voets, J. P., 66
Vogel, O. A., 380
Vogt, M., 341
Volkers, W. S., 100
Vollenweider, R. A., 147
von Bismarck, R., 421., 428
von Bredow, K., 385
von Dennfer, D., 347
von Euler, H., 2
von Guttenberg, H., 134, 135, 141, 143, 424, 428
von Hausen, S., 9
von Hoehnel, F., 272
Von Maltzahn, K. E., 399
Von Saltza, M. H., 396, 397, 403
Von Wettstein, D., 15, 251
Vos, J. J., 168, 169
Voskresenskaya, N. P., 277
Voznesenskii, V. L., 142
Vyskrebentseva, E. I., 37

W

Wadkins, C. L., 14, 25, 180
Wadleigh, C. H., 98, 99, 280, 282, 283, 284
Wadsworth, R. M., 140
Waelsch, H., 4
Wagenaar, S., 312
Waggoner, P. E., 441, 450, 454, 456
Wagle, S. R., 129
Wahlroos, O., 4, 67
Wain, R. L., 77, 249, 345, 346, 347, 348, 350, 355, 356, 359, 361, 362
Wainio, W. W., 23, 24
Wainwright, S. D., 114
Walhood, V. T., 372
Walker, D. A., 20, 76, 180, 182
Walker, D. R., 381, 383
Walker, G. C., 66, 69, 70, 72
Walker, J. C., 456
Walker, W. S., 51
Wallace, A., 63, 75, 76, 77, 102, 107
Wallace, T., 63
Walter, H., 133, 136, 139, 140, 141, 146, 149, 265
Wang, C. H., 256, 259
Wang, Li Chuan, 66
Wang, Y. L., 78
Wanta, R. C., 431
Warburg, O., 156, 157, 185, 334
Wardlaw, C. W., 265, 400
Wardrop, A. B., 35, 36, 40, 44, 45, 46, 47, 48, 49
Wareing, P. F., 293, 306, 307, 313, 350, 354, 382, 386
Wargel, C. J., 372, 378
Waris, H., 10
Warren, C. K., 219, 235
Wasserman, L., 309
Wassink, E. C., 133, 136, 137, 144, 186, 293
Watanabe, F. S., 92, 95
Watson, D. J., 145, 146, 149
Watson, J. D., 126, 127
Watson, M. L., 14, 16
Wattiaux, R., 29
Watts, J. W., 332
Waygood, E. R., 80, 460
Weatherley, P. E., 209, 277
Weaver, M. L., 458
Weaver, R. J., 373, 377, 382, 383, 389
Webb, B. K., 372, 378
Webb, E. C., 104, 105
Webb, J. A., 8
Webb, R. A., 84
Weber, F., 25, 246
Weber, G., 170
Weber, M. M., 26
Weber, R. P., 350
Webster, G. C., 113-32; 4, 73, 114, 115, 118, 119, 120, 123, 125, 127, 196, 207, 208, 251, 253, 254, 255
Webster, L. T., Jr., 119
Wedding, R. T., 438, 441, 445
Weedon, B. C. L., 219, 234, 235
Wei, L. S., 95
Weibel, R. O., 384
Weigl, J. W., 158
Weijer, J., 386
Weill, J. D., 254
Weinbach, E. C., 19
Weinstein, L. H., 76, 77, 256, 257
Weiss, J., 168
Welch, L. F., 92
Wellensiek, S. J., 293, 295, 299, 306, 310, 312, 313, 317, 318
Weller, L. E., 345, 348, 374, 375, 379, 386, 389
Weller, L. W., 355
Wells, N., 92
Went, F. W., 236, 309, 311, 345, 435, 436, 443
Went, H. A., 335
Wessel, G., 64
Wessels, J. S. C., 164
West, C. A., 312, 369
West, G. B., 345, 347
Westing, A. H., 376
Wetmore, R. H., 320, 347, 361
Whaley, W. G., 38, 39, 48, 251, 255, 256, 340
Whaling, M. L., 357
Whallon, J., 401
Wharton, D. C., 456
Whatley, F. R., 78, 201, 255
Wheeler, A. W., 350, 353, 355
Whightman, F., 77
Whistler, R. L., 35, 36, 37
White, E. P., 347
Whitfield, P. R., 122
Whittingham, C. P., 163, 178
Whittington, W. J., 83
Whyte, R. O., 293
Wickson, M., 399, 405, 406
Widmer, C., 23, 24, 25, 27
Wiebe, G. A., 303
Wiebe, H. H., 75, 107
Wiersma, D., 281
Wiersum, L. K., 93, 95, 97, 98
Wightman, F., 55, 346, 350, 355, 356, 359, 361, 362
Wiklander, L., 92, 107
Wilda, E. F., 135
Wildman, S. G., 361
Wildy, P., 335
Wilhelm, S., 455
Wilkins, M. H. F., 126
Wilkins, M. J., 251
Williams, W. R., 273
Willmer, E. N., 5,
Willmer, J. S., 231
Willstätter, R., 133, 135
Wilson, C. C., 140, 267, 268, 269, 277
Wilson, C. M., 37, 55
Wilson, G. B., 329, 337
Wilson, G. L., 149
Wilson, K., 47

Wilson, L. G., 208
Wilson, P. W., 3, 7, 24, 65, 66
Wilson, S. B., 64, 66
Wiltshire, G. H., 250
Wimber, D., 328
Winfield, M. E., 65, 83
Wingard, S. A., 454
Winkler, E., 134, 135, 139, 140, 141
Winneberger, J. H., 266
Winstantley, D. L., 411
Winters, H. F., 282
Wirth, P., 37
Wiss, O., 25
Withrow, A. P., 317
Withrow, R. B., 293, 294, 298, 304, 305, 308, 317
Witt, H. T., 81, 162, 164, 169
Witting, L. A., 224
Wittreich, P. E., 222
Wittwer, S. H., 345, 346, 348, 355, 369, 370, 371, 374, 375, 376, 378, 379, 380, 381, 383, 384, 385, 386, 338, 389
Wojtczak, A. B., 24
Wojtczak, L., 24
Wolf, F. A., 282
Wolf, F. T., 361, 378
Wolf, M., 142
Wolfe, M., 64
Wolfe, R. W., 240
Wolfrom, M. L., 40, 46
Wolken, J. J., 155
Wollman, E. L., 341
Woltz, W. G., 92
Wong, K. K., 119
Wood, H. G., 256
Wood, J. G., 255, 281
Wood, R. K. S., 454, 457
Woodard, J. W., 330, 331
Woodhams, D. H., 280, 281
Woods, P. S., 328, 331, 333
Woolf, B., 1
Woolley, J. T., 64
Work, T. S., 19, 28, 118
Worsham, A. D., 401
Wosilait, W. D., 83
Wright, B. C., 93
Wright, B. E., 257
Wright, G. M., 380
Wright, K. E., 99
Wright, L. D., 221
Wright, M. A., 66
Wright, S. T. C., 254, 352
Wu, P. H. L., 44
Wu, T., 197, 199, 211
Wuhrmann, J. J., 225
Wurgler, W., 349
Würsch, J., 221
Wyman, J., 95, 104
Wynn-Parry, D., 357

Y

Yager, R. E., 253
Yamada, M., 380
Yamada, N., 148, 435
Yamaguchi, M., 219, 251
Yamaki, T., 346, 356, 361, 369, 370, 380
Yamamoto, Y., 207, 249, 250
Yamanaka, T., 70
Yamasaki, K., 67
Yamashina, I., 5
Yamazaki, K., 386
Yang, Shang-Fa, 257
Yarosh, N. P., 281
Yarwood, C. E., 446
Yemm, E. W., 196, 207, 250
Yentsch, C. S., 139, 141, 145
Yin, F. H., 127
Yocum, C. S., 82, 203
Yoda, S., 54
Yokoyama, H., 221, 222, 225, 236
Yoneyama, Y., 19
Yoshida, A., 123
Yoshida, M., 374
Yoshikawa, H., 19
Yoshimura, S., 143
Young, J. L., 248, 249, 254
Young, P. A., 452
Young, R. E., 77
Young, R. H., 77
Younger, V. B., 371
Younis, A. F., 414, 426, 427
Youssef, E., 347, 352, 355

Z

Zabin, I., 208, 220, 222, 223, 236, 248
Zachau, H. G., 121
Zähner, H., 456, 459
Zaitlin, M., 432, 434, 439, 440
Zalensky, O. V., 133, 139, 141, 142
Zalik, S., 359
Zalkin, H., 25
Zalokar, M., 237, 253
Zamecnik, P. C., 116, 119, 120, 121, 122, 123, 251
Zandee, D. I., 410, 420
Zaugg, W. S., 178, 179
Zavarzin, G. A., 74
Zebrun, W., 15
Zechmeister, L., 236
Zeevaart, J. A. D., 301, 303, 309, 317, 318, 319
Zeller, P., 219
Zeuthen, E., 334, 335
Zholkevich, V. N., 281, 282
Ziegenspeck, H., 246, 253, 254
Zieger, G., 162
Ziegler, D. M., 14, 15, 23, 25
Ziegler, H., 421
Zillig, W., 118, 121
Zimmerman, P. W., 431, 441
Zimmermann, W., 140, 414
Zink, F. W., 375
Zinke, H., 433
Zubay, G., 126
Zucker, M., 71

SUBJECT INDEX

A

Abscission
  gibberellin and, 372
  zones, 253
Absorption rate, 267-69
Actinic light studies, 167
Action spectra, 171
Active entry, 100
Active ion accumulation, 100-7
ADP
  in mitochondria, 17, 19
  role of, 206
Adventitious root formation, 456
Ageing, 18
  calcium and, 246
  cell permeability, 246
  cell wall and, 252
  chemical changes, 257
  chloroplasts and, 255
  cytoplasmic membrane and, 252
  2,4-D and, 252
  general theories, 245-47
  metabolic pathway changes, 256
  radiation and, 246
  vacuole and, 255
Air pollution, 431-47
  damage susceptibility, 434
  oxidants, 432-40
Alanine, 119, 120
  synthesis, 2, 3, 5
Algae
  cell wall, 50
  lamellar systems, 156
  mineral requirements, 64, 66
  mitosis in, 336
  photosynthesis in, 133
  photosynthetic yields, 136, 139
Alkaloids and water deficit, 282
Amides in nitrate reduction, 73
Amination, reductive, 2, 3
Amino acid
  activation, 118-19
  enzymes and, 1-10
  incorporation into protein, 116
  morphogenetic effect of, 10
  phosphorylation in, 196, 207
  polypeptide formation, 122-25
  primary vs. secondary, 2, 7
  ribosomes, 125
  root feeding of, 9
  sRNA and, 122
  synthesis, 1-10
  transamination and, 7-8
  transfer, 123
  see also Protein synthesis
Amino acid-acceptor RNA, 120-22
Ammonia in nitrate reduction, 72-73
Amylase, 117
Anaerobiasis and phosphorylation, 213
Annual dry weight yields, 136
Anthers, 341
Anthocyanin and kinetin, 402
Antiauxin, 313
Antibiotics as cell division inhibitors, 337
Antigeotropism, 428
Antimetabolites, 314
Antimycin, 74
Antiozonants, 445
Aquatic plants, photosynthesis in, 133, 140
Araban, 43
Arsenate as flower inhibitor, 314
Ascites tumor cells, 337
Ascorbate oxidation, 203
Ascorbic acid
  cytochrome and, 82
  photooxidation, 179
  plant growth and, 358
Ascorbigen, 347, 354, 357-59
Aspartase, 1
Atmospheric variables, 286
ATP
  in mitochondria, 15, 16, 17, 19
  synthesis, 119
Auxin
  cell wall plasticity and, 52-53
  changes in wilt disease, 464
  flowering hormone and, 311
  flowering inhibition by, 313
  free and bound, 356
  genetics of, 349
  geotropism and, 410
  gibberellin and, 385
  IAA precursor, 355
  in abnormal plants, 347-49
  indole, 345-63
  in normal plants, 345
  in wilt symptoms, 456
  kinetin relationship, 405
  light and, 348
  nutrients and, 349
  physiology, 345
  reagent tests, 350
  senescence and, 246
  tumors and, 347
  utilization of, 355-56
  virus infection and, 348
Avocado storage, 257
Azide as flower inhibitor, 314
Azotobacter, hydroxylamine in, 6, 7

B

Bacillus megaterium, protein synthesis in, 114
Bacteria
  carotenoid synthesis in, 229
  cellulose production in, 41
  chlorophylls in, 168
  nucleic acid in, 332
  phosphorylation in, 27
  photosynthetic, 186, 237, 239
  purple, 238
  wilt disease in, 457
Bacteriochlorophyll, 239
Bicarbonate and chlorosis, 75-77
Biological time measurement, 304
Boron requirement, 64, 83
Branching, 50
Bromine requirement, 64
Bud
  elongation and kinetin, 399
  flowering and, 318
  formation and kinetin, 398

C

Cadmium requirement, 81
Calcareous soils, 92
Calcium
  ageing and, 246
  effect on cell wall, 54
  form in soils, 92
  inhibitor of ion uptake, 104
  mitochondrial, 19
  requirement, 65
Cancer cells, 334
Capsanthin, 235
Capsorubin, 235
Carbohydrate
  boron and, 83

interconversion of, 205-6
metabolic changes, 460
non-cellulose, 42-44
Carbon dioxide
carotenoids and, 225, 226
photosynthesis and, 279
supply effect, 139
Carbon dioxide fixation
chlorosis and, 76
gibberellin and, 377
photosynthetic, 135
Carbon increase, 135
Carotenes
hydroxylated, 232
Carotenoids
biosynthesis of, 219-40
chemistry of, 219
CO_2 incorporation, 225
cyclic vs. acyclic, 230
epoxides, 235
formation in cell-free systems, 236-37
function of, 237-40
hydroxylated, 232
illumination and, 160
leucine and, 224
methoxylated, 233
oxidation, 235
oxygen function, 232-36
photophosphorylation, 238
photosensitization protector, 239
photosynthesis in, 237
precursor cyclization, 230
40-C precursors of, 227-28
Cell
culture, 340
division, 279
see also Cell division
enlargement and kinetin, 397-98
expansion, 51
non-growing, 332
permeability and ageing, 246
sub-cellular changes, 251
turgor pressure, 278
wall, see Cell wall
work during senescence, 247-51
Cell division
cancerous, 334
chemical agents and, 337, 339
cycle, 328-35
inhibitors of, 210
kinetin and, 397-98
radiation and, 336
spindle, 339-40
stages, 333-35
temperature and, 335
Cell-free systems
carotenoid formation in, 236-37
phosphorylation in, 196
protein synthesis in, 114
Cellulose
structure of, 35-36
synthesis of, 40-42
Cell wall
ageing and, 252
branching, 50
chemical composition of, 35-40
chemical interactions in, 39-40
deposition sites, 47-51
extension of, 47-51
growth control, 51-58
microfibrils in, 44-47
middle lamella of, 39
multinet structure, 44-45, 48
non-cellulose carbohydrates, 42-44
physiology of, 35-58
plasticization, 52-58
polysaccharides in, 35-36, 40-44
quantitative studies of, 36-38
stress in, 49-50
structure of, 35-58
"thinning," 52
water loss from, 273-74
Centrioles, 339
Centromeres, 339
Chelates, 75
Chelation in mitochondria, 17
Cherry and gibberellin, 374
Chitin structure, 36
Chloramphenicol, 117
Chlorella electron spin studies, 158-59
Chlorine requirement, 64
Chlorophylls, 166-68
Chloroplasts
ageing and, 255
carotenoids in, 237
lipid extraction in, 25
membrane function, 15
nucleic acid in, 255
oxidant damage to, 435
protein synthesis in, 128, 255
reactions, 178-87
RNA synthesis, 333
structure, 155-56
Chlorosis from oxidants, 433, 435
Cholesterol precursor, 222
Chromatophore, 238
Chromogenic spots, 348
Chromosomes in interphase, 328, 331
Circadian rhythms, 307
Cis-isomers, 235
Citric acid cycle in mitochondria, 20
Citrus fruits, 257
Cleaving eggs, 337
Climatic variables, 286
Cobalt
flowering and, 309
requirement, 64, 66
Coffee and gibberellin, 372
Cohesion concept, 267
Collenchyma microfibrils, 46, 47
Conifers and gibberellin, 377
Continuous percolation technique, 94
Copper
in cytochrome oxidase, 23
requirement, 66, 70, 71, 82-83
Corn
gibberellin and, 371
mineral requirements, 98
yield and water, 282
Cotton
gibberellin and, 372
wilt, 449
Coumarin, 388
Cristae, 15, 19
Crop profiles, 146
Crop yields
gibberellin and, 371
water deficit and, 282
Crystallinity of cellulose, 36
Cyanide as flower inhibitor, 314
Cytochrome
ascorbic acid and, 82
extraction table, 22
incidence in plants, 78
nitrate reduction and, 67-69
oxidase, 22-23
photosynthesis role, 165
synthesis, 115-16
Cytophotometry, 330
Cytoplasmic ground substance, 255-56
Cytoplasmic membrane and ageing, 252

D

2,4-D as ageing inhibitor, 252
Dark periods, 303
Decapitation effect, 425
Defoliation, 456
Desert plants, 139
Detergent effect on mitochondria, 16
Diakinesis, 333
DNA
in cell division, 328-29
synthesis and radiation, 336
Donnan effects, 107
Dormancy
breaking and kinetin, 400
gibberellin and, 380
Double membrane phenomenon, 14-15

Dwarfism, 380, 456

E

Ecology, 138
  biological time, 304
  photosynthetic measurement of community, 148
Electron spin resonance studies, 158-60
Electron transport in mitochondria, 13, 20-24
Endogenous timing, 307
Endoplasmic reticulum, 253
Endosperm, glyoxalate cycle, 256
Energy requirement for transpiration, 266
Enhancement effect, 171
Enolase, 82
Enzymes
  abscission and, 253
  activating amino acids, 118
  ageing and, 250
  amino acid transfer, 123
  carotenoid synthesis, 236
  cell-free, 1
  cellulose synthesis, 41-44
  cytochromes and, 78
  gibberellin effect on, 378-79
  liver, 224
  manganese effect, 80
  metal requirement, 73
  mitochondrial, 13, 16, 18, 26
  phosphate fractionation studies, 197
  stability, 118
  synthesis of protein, 114-17
  synthesis by ribosome, 249
  systems and amino acid synthesis, 1-10
  systems and nitrogen fixation, 65-74
  yeast, 224
Eosin, 428
Epidermal cells, 47
Epinasty, 456
E. P. R., 83
Equilibrium sorption reactions, 95
Ethylene
  as autocatalyst, 450
  biogenesis of, 257-60
  damage to plants, 443
Ethyl indole-3-acetate, 346
Evergreens, 139
Exogenous timing, 307
Exudation, 269

F

Fat metabolism, phosphorylation, 208
Fertility and soil moisture, 285
Fertilizers, 379
Fibroblasts, 18, 337
Florigen, 294
Flowering
  antimetabolites and, 314
  clock types, 307-8
  cobalt and, 309
  hormone action, 319
  hormone synthesis, 309-16
  inhibition reversals, 315
  inhibitors of, 309-10
  kinetics of pigment system, 297-304
  light effect after dark, 316-17
  light inhibition of, 300
  moisture and, 282
  photoperiodism and, 293-321
  phytochrome forms and, 303
  pigment biochemistry and, 304
  process, 294-96
  timing of, 304-9
  translocation of hormone, 317
Flowers
  gibberellin and, 376
  petal carotenoids, 235
Fluorescence clearing in wilt disease, 455
Fluoride
  damage to plants, 443
  flower inhibitor, 314
Formic acid, 4, 432
"free" space, 99
"frenching" disease, 10
Freundlich isotherm, 95
Fruit
  cis-isomers in, 236
  ethylene and, 257
  function for seed, 248
  ripening, 257
  settling and gibberellin, 374
  water deficit in, 268
Functional lipid molecules, 25
Fungus
  auxin in, 348
  cell wall of, 36
  mineral requirement, 64
  tumor in, 347
  see also Wilt
Fusaric acid, 449, 459

G

Gas chromatography, 257
Genetics
  auxin, 349
  carotenoid, 230
  tomato, 236
Geoelectric response, 421
Geotropism
  antigeotropism, 428
  course of, 412-15
  growth regulators and, 415
  IAA and, 418
  light effect, 428
  methods of study, 410-12
  presentation time, 411
  roots and shoots, 409-29
  stimulation, 420-28
Germination
  boron effects on, 84
  gibberellin and, 378, 381
  radiation sensitivity and, 246
  seedlings, 69
Gibberellins, 369-89
  acids, 369-70
  acyls, 370
  age inhibitor, 252
  auxin and, 385
  crop yields, 371-77
  dormancy and, 380
  dosages, 389
  effect on assimilation, 377-78
  effect on translocation, 377-78
  enzymes and, 378-79
  esters, 370-71
  fertilizers and, 379
  flowering and, 312
  germination and, 381
  growth-retarding, 387-88
  nodulation and, 379
  photoperiod and, 384
  pollen and, 379
  purine derivatives, 387
  respiration and, 378
  salts, 370
  surface-active agents, 371
  terminology, 369
  vernalization and, 383
Glucose in cellulose synthesis, 40
Glucoside synthesis, 4
Glutamic acid origin, 7
Glutamine synthesis, 2, 3
Glutathione as wound healer, 451
Glycine, 119
Glycolysis
  aerobic, 334
  phosphorylation in, 207, 211
Grain sorghum and gibberellin, 372
Granules, carotenoids in, 15
Grapes and gibberellin, 373
Graphical analyses, 103-5
Grasses and amino acids, 9
Green plants, amino acid synthesis in, 1-10
Growing tip water deficit, 268
Growth

amino acid and, 9
autotrophic, 81
control by cell wall, 51-58
geotropism and, 427
heterotrophic, 81
leaf units, 149
oxidant effect on, 435-38
phosphorylation and, 210
plasticity and, 53
regulators and geotropism, 415-20
trace metals and, 77
turgor pressure role in, 278
water deficit and, 282, 284
Guard cells, 50
Gums, 457

H

Hair cells, 46
Halophytes, photosynthesis in, 142
Hematin, 78
Hemicelluloses, 36
Hemoglobin, 66
synthesis, 115
Hexene, 438
Hexokinase, 81
Higher plants, phosphorylation in, 195-216
Histones, 330
Hops and gibberellin, 373
Hormone
biochemistry, 312
florigen, 294
for flowering, 309-16, 319
level and ageing, 253
time of synthesis, 310
translocation, 318
Host metabolic derangement, 458
Hydroxamic acids, 4
Hydroxylamine
amino acid synthesis and, 3, 4, 6
"bound," 4-5, 6
metal requirement, 72
Hyponitrite, 71

I

IAA
geotropism and, 418
see also Auxin
IAN, 346
Illumination, 134
Incipient drying hypothesis, 274
Indole auxins, 345-63
interconversions of, 359-63
precursors, 347
unidentified, 349-55
Indole-3-acetaldehyde, 346
Infrared absorption analysis, 134
Inhibition
of ageing, 18
competitive, 106
of electron transport, 20
of ion uptake, 103, 209
of nitrification, 74
of protein synthesis, 117
Internodal cells, 46
Interphase, 328, 335-39
Ion
accumulation in roots, 108
active accumulation, 100-7
biological constants, 101
carrier constants, 101-2
diffusion, 99-100
entrance into root, 99-100
movement in soil, 98-99
movement to tops, 107-9
release by soil, 94, 97
uptake, see Ion uptake
ionic interactions, 103
Ion uptake
active, 107-8
competitive inhibition, 106
graphical analysis, 103-5
inhibitors of, 209
passive, 107
phosphorylation and, 209
by root, 97
soil-plant relationship in, 91-109
solid-phase effects, 107
Iron
chlorosis, 75
metabolism, 75-78
requirements, 65-68, 70-71, 73, 75
Islands of synthesis theory, 48
Isoleucine, 119

K

Keto acids in amino acid synthesis, 1-10
Keto-carotenoids, 234-35
Kinetin
as age inhibitor, 252
auxin relationship, 405
biochemical effects, 401
biological effects, 397-402
buds and, 399
cell division and, 338
discovery of, 395-97
dormancy breaking and, 400
leaf growth and, 400
leaf protein and, 401
light effects and, 406
plant growth and, 395-407
related compounds, 395-407
root growth and, 399
shoot initiation, 398
similar compounds, 403-6
structural requirements, 402
structure of, 397
Kinetochores, 339
Krebs cycle, 202

L

Lamellae, 155
Langmuir isotherm, 95, 96
Lanosterol, 233
Latitude and photosynthesis, 135
Lawn grass, 371
Leaf
age and air damage, 434
area index, 145
assimilates of, 134
boron in, 84
carotene in, 235
flowering inhibitor in, 310
growth and kinetin, 400
inhibition of flowering in, 318
light experiments on, 300
non-stomatal water loss, 273, 275
ozone injury symptoms, 440-41
photosynthetic studies, 136
plasmolysis, 458
protein content and kinetin, 401
radiation resistance, 246
senescence in, 252
temperature and hormone synthesis, 311
water content, 270, 272-73
water deficit in, 268
Lecithinase as uncoupler, 24
Legume root nodules, 6
Leptotene, 333, 341
Lettuce deheading, 375
Leucine, 119, 120, 122
carotenogenesis and, 224
metabolism, 222
Lichens, 139
Light
auxin level and, 348
blue effects, 294
effect after dark, 316-17
effect on cell division, 336
effect on geotropism, 428
interruption effects, 294, 299
kinetin effect, 406
quality used, 300
quantities used, 299
red effects, 294
Light-minus-dark-spectra, 160-69
Light-saturation effects, 137-38
Lipids
extraction, 25
in cell wall, 38
in electron transport, 13
phosphorylation and, 196
Lipoprotein

layers, 14
see also Mitochondria
Lithium, 104
long-day plants, 294
Lutein, 235
Lycomarasmin, 458
Lycopene, 220
production of, 228
Lyosomes, 28
Lyotropic series relationships, 107

M

Magnesium
requirement, 73
role in amino acid synthesis, 117
Malonic acid, 77
Manganese
form in soils, 92
requirement, 72, 80
Marine photosynthesis, 142-43
Measurement problems, 276
Meiosis
physiology of, 327-42
RNA synthesis in, 333
Membranes
mitochondrial, 14-15
permeability, 15
Meristem, mitochondria in, 19
Metabolic pathways in ageing, 256
Metabolism
during mitosis, 334
life duration and, 246
respiratory, 196
Metallo-enzymes, 81, 85
Methionine, 119
Methods
field photosynthetic studies, 134
phosphate metabolism studies, 196-97
Mevalonic acid, 222, 223
Micelles, 339
Michaelis-Menten constants, 101-2
Microfibrils, 35-36
arrangement in cell wall, 44-47
Microorganisms
amino acid synthesis in, 1, 5
carotenogenesis in, 229
non-photosynthetic, 240
Microsomes and protein synthesis, 116
Microsporocytes, 331
Microvilli, 15
Middle lamella composition, 38-39
Mineral nutrients, 63-85
Mineralogy, 92-93
Mitochondria
biogenesis of, 251
citric acid cycle in, 20
composition of, 26-28
DNA in, 337
electron transport in, 13, 20-24
enzymes in, 26
ethylene synthesis in, 258
formation of, 18-19
function of, 20-29
inhibitors, 250
life span and, 247
lipid molecules in, 25
metabolism, 13
metal chelation in, 17
nucleic acid in, 27
ozone effect on, 442
phosphorylation, 16-18, 25-26
postharvest tissues, 250
protein synthesis in, 19, 115, 128
purification, 28-29
pyridine nucleotides in, 19
senescent cells, 247, 248
structure, 14-20
swelling, 15-16
uncouplers, 250
Mitosis
antibiotic and, 337
energy requirement during, 335
inhibition of, 334
physiology of, 327-42
Molybdenum
form in soils, 92
requirement, 64-66, 68, 69, 74, 78
Morphogenesis, 10, 319
Mosaic growth, 48
Mosses, 139
Mutation, 246
Mycelial growth, 69
Myelin figures, 14

N

Nicotine and water deficit, 282
Nitrate
reductase, 67
reduction, 3, 72-73
requirements, 66-70
Nitric oxide, 71
Nitrification metal requirement, 73-74
Nitrite requirement, 70-71
Nitrogen
in cell wall, 38
deficiency and enzyme action, 6
effects on air damage to plant, 434
form in soils, 92
metabolic changes, 460
metabolism, 65-74
requirement, 64, 65-74
uptake in root, 8-9
water deficit and, 281
wilt disease and, 454
Nitrogen dioxide damage, 439
Nitrogen fixation, 65-66
amino acid synthesis, 3, 6
anaerobic, 6
metals and, 66
Nitrogen mustard application, 246
Nitrous oxide, 71
Nodulation, gibberellin and, 379
Nodule bacteria, 64, 70
Necrosis, 83, 456
Nematodes and wilt disease, 452
Neurosporene, 236
Nuclei, 253
protein synthesis in, 128
Nucleic acid, 27
phosphorylation and, 196
Nucleoli, 255
Nucleotides, 121, 332
Nucleus, 240
ageing and, 246, 254
biological timing and, 309
DNA in, 328
isolation of, 255
meiosis and, 328
Nutrient
concentration in soil, 93
minor mineral, 63-85
uptake and water deficit, 282-83

O

Oak wilt, 457
"Opening phenomena," 16
Orange and gibberellin, 375
Organ culture, 340
Organic acid and chlorosis, 76
Osmosis, 51
Osmotic effects, 278-79
Oxidants, 432-40
pathology of damage, 434-35
Oxidative phosphorylation in senescent cells, 247
Oxine formation, 4, 5
Oxygen
consumption in cell division, 334
inhibition by, 213
isotope exchange, 13-14
role in swelling, 17
transfer, 119
uptake and ageing, 249
Ozone, 440-44
antiozonants, 445
geographical occurrence of, 442
injury symptoms, 440-41

physiological effects, 441-42
Ozone-olefin dark reactions, 432-34

P

Parenchyma microfibrils, 45, 47
Parthenocarpy and gibberellin, 374
Passive entry, 99
Peach
  gibberellin and, 374
  ripening, 257
Pea ribosomes, 114
Pectic substances in cell wall, 39
Pectin
  growth and, 56
  in oats, 38
  synthesis, 44, 205
Pectolytic enzymes and wilt, 454
Pentanoid rings, 219
Pentose phosphate pathway, 203
Peptides in nitrate reduction, 73
Peroxyacetyl nitrite, 439
Petunia and smog, 437
Phalloidin, 428
Phloem, boron effect on, 83
Phosphate
  bonds, 195
  compounds in higher plants, 197
  metabolism studies, 196-97
  physiology and, 208-10
  tissue levels, 198
  transfer potential, 204
  turnover, 199-201
  use of, 204-8
Phosphate-acceptor levels, 212
Phospholipid synthesis, 19
Phosphorus
  absorption data, 95-96
  concentration in soil, 100
  form in soil, 92
  metabolism, 78-80
Phosphorylated metabolites, 200
Phosphorylation
  ageing and, 249
  amino acids and, 196, 207
  anaerobiosis and, 213
  carbohydrate interconversion, 205
  cell-free system, 196
  factors in, 25-26
  fat metabolism and, 208
  glycolysis and, 207
  growth and, 210
  higher plant, 195-216
  inhibitors of, 20
  lipids and, 196
  mitochondrial, 15, 16-18, 201-2
  nucleic acid, 196
  photosynthetic, 196
  primary, 201-4
  respiration and, 196, 210
  sugar, 196
  sulfur metabolism and, 208
  uncoupling, 212, 214
Photochemical air pollution
  control of, 446
  damage to plants, 431-47
Photochemistry reciprocity, 298
Photomorphogenetic processes, 297
Photooxidations, 178
Photoperiodism, 298, 304-9
  flowering and, 293-321
  gibberellin and, 384
  pigment system and, 296-304
  water cycle, 268
Photophosphorylation, 180-87, 238
Photosensitivity, 239-40
Photosynthesis, 155-89
  action spectra, 171
  air pollutants and, 438
  bacterial, 168, 186
  carbon dioxide supply and, 139-40
  carotenoid, 237
  chlorophylls, 166-68
  chloroplast structure, 155-56
  cover density, 145-46
  depth-integration of rates, 146
  diurnal changes, 138-39, 141
  electron spin resonance studies, 158-60
  enhancement effect, 171
  light minus dark spectra, 160-69
  light-saturation, 137-38
  manganese effect, 81
  natural, 133-49
  phosphorylation and, 196
  pigments, 169-71
  primary reactions, 187-89
  primary reductant, 169
  quantitative relations, 172
  quantum yield, 156-57
  R factor, 157-58
  rate variation, 138-42
  seasonal variation, 139, 140
  shaded and exposed, 142
  sources of information, 134-35
  stomata and, 140
  water deficit and, 277, 279-80
  wilting and, 280
  yields, 135-37
Phycobilin, 297
Phytochrome forms, 297, 303
Phytoene, 227, 230-31
Phytofluene, 236
Phytotoxicant, 433
Pigment
  biochemistry, 304
  conversion, 234
  lamellar system and, 155
  photosynthetic, 146, 169-71
  system, 296-304
  see also Carotenoids
Pit fields synthesis theory, 48
Plagiotropic organs, 409, 413
Plankton photosynthesis, 133, 139, 145
Plant
  abnormal, 347-49
  auxin, in, 345-49
  composition and water deficit, 280
  micro-universe, 265
  photochemical air pollution damage, 431-47
Plasma
  membrane impairment, 460
  papillae theory, 48
Plasticity of cell wall, 52-56
Pollen
  gibberellin and, 379
  wall growth, 48
Polymerization of isopentenyl pyrophosphate, 226-27
5-C Polymerizing unit, 220
Polypeptide
  chain changes, 125
  formation, 122
  release, 125
Polysaccharide
  biosynthesis, 40-44
  in cell wall, 35-36
Post-harvest tissue life span, 247
Potassium form in soils, 92
Primary reductant, 169
Primary wall structure, 46-47
Protein
  amino acid incorporation, 116
  hydration of, 277
  membrane structure, 15
  metabolism
    in ageing, 250
    water deficit and, 281
  synthesis, 113-29
    catalyst for, 115
    cell-free, 114
    endosperm, 249
    enzymatic, 114-17

flowering hormone and, 314
inhibitors of, 117
mitochondrial, 19
senescence and, 247
see also Meiosis
Proteolysis and water deficit, 281
Protists, 240
Protoplasmic hydration, 277
Protoplasmic tip growth, 48
Purification methods, 28-29
Purple bacteria, 233
Pyridine nucleotides, 19, 199
light and, 158
Pyrophosphate exchange reactions, 119

Q

Quantitative relations, 172
Quantum yield, 156-57
Quercitin, 314

R

Radiation
effects, 84
injury, 245
mitosis and, 336
Radioactive amino acids, 115
Radioactive tracers in ethylene studies, 258
Reciprocity, 298
Reductive amination, 2
Relative turgidity, 277
Relaxation oscillator, 308
Resonance, inductive, 238
Respiration
air pollutants and, 438
cancer cells, 334
changes in wilt, 461
climacteric rise in, 248
gibberellin and, 378
mitochondrial, 21
rate in water deficit, 281
vitamin E requirement, 25
Respiratory metabolism and phosphorylation, 210
R factor, 157-58
Rhizosphere, 452
Rhodopin, 233
Rhodovibrin, 234
Ribonuclease, 27
ageing, 251
cell division and, 337
inhibition by, 117
Ribonucleic acid, 28
Ribonucleoprotein particles, 253
ageing and, 254
Ribosomal RNA, 124
Ribosome, 253
protein synthesis and, 114-16
structure, 125-27
Ripening, 247
ethylene and, 257
RNA, 124
kinetin and, 401
synthesis, see Mitosis
Root
elongation, 267
geotropism in, 409-29
growth and kinetin, 399
infection factors, 451-55
ion uptake, 97, 99
nitrogen uptake in, 8-9
water absorption, 267
wound hormones, 451
Root-ion complexes, 101-2
Root tip
cell division in, 329
mitochondria in, 19
Roses, changes in cutting, 257
Rotifers, 246
Rubidium, 98

S

Scopoletin, 456
Season, photosynthesis and, 135
Seed, gibberellin and, 381, 383-84
Seedling
gibberellin and, 376
mitochondria in, 19
Senescence
biochemistry of, 245
cellular work during, 247-51
respiration during, 247-51
subcellular changes, 251
water deficit and, 282
see also Ageing
Serine, 119
Shade plants, 144
Shoot
geotropism in, 415
initiation and kinetin, 398
ion transport, 107
short-day plants, 294
Skatole, 347
Smog
cities studied, 443
effects, 431
Sodium citrate and longevity, 246
Soil
acidity and wilt disease, 452
chemical identification, 92-93
ion saturation, 97
mineralogical identification, 92-93
moisture effects, 269
moisture and plant water deficits, 283-84
solid phase, 92-97
water movement, 98-99
see also Soil-plant system
Soil-plant system
dynamics of, 91-92
ion uptake and, 91-109
nutrient solutions, 92-93
solid soil phase, 92-97
solution movement, 97-99
root entrance, 99-100
Solar energy utilization, 136
Solubility tests on soil, 92
Solute uptake and phosphorylation, 209
Soybean and gibberellin, 372
Spindle, 339-40
proteins, 335
Spirilloxanthin, 229
Squalene synthesis, 226-27, 233
sRNA, 120-22
Starch
conversion in water deficit, 280
role in geotropism, 421
Statoliths, 421, 422
Stoichiometry, 52
Stomata
photosynthesis and, 140
water loss control by, 272
Stomatal aperture and water loss, 275
Strawberry and gibberellin, 375
Strontium, 95
Stunting, 456
Subcellular changes, in ageing, 251-56
Sucrose synthesis, 250
Sugar beet and gibberellin, 373
Sugar cane and gibberellin, 372
Sugars and phosphorylation, 196
Sulfhydryl and cell division, 335
Sulfur metabolism, phosphorylation in, 208
Swelling phenomena, 15-18
Synergism, 356-57

T

Techniques, see Methods
Temperature
cell division and, 335
day length response and, 295
flowering control and, 309
growth and, 53
life span and, 247
photosynthesis and, 138-39
wilt disease and, 452
Threonine, 119
Thymidine, 328
Time effects, 173
Tobacco

amino acids and, 10
gibberellin and, 373
Tocopherol, 25
Tomato
air pollutants and, 437
carotenoids in, 220, 236
gibberellin and, 375
lycopene in, 228
ripening, 247
wilt disease in, 455
Tonoplast, 252
Trace metal requirement, 64, 65
Tracheids, microfibrils, 45
Transamination, 7, 8
Translocation, boron and, 87
Transpiration, 98
rate of, 267-69
stream, 266
Traumatic acid, 451
Tree-crown photosynthesis, 143
Trees
gibberellin and, 376
growth inhibition, 436
photosynthesis in, 133
water level in, 268
Tropical forest, 140
Tryptamine, 347
Tryptophan, 81, 119
occurrence, 347
synthesis of, 359
Tumor
auxin and, 347
inhibition and, 337
Tungstate requirement, 74
Turgor, 269
Tyloses, 457
Tyrosine, 119

U

Uncouplers, 212, 214
Uridine polyphosphate role, 206

V

Vacuole
ageing and, 255
isolation of, 255
Valine, 119, 120
Vanadium requirement, 64
van der Waal's forces, 39
Vascular browning, 457
Vascular occlusions, 458
Vegetable yield and gibberellin, 375-76
Vein clearing in wilt disease, 455
Vernalization, 293
gibberellin and, 383
Violazanthin, 235
Virus infection and auxin level, 348
Vitamin
B, 66
E, 25
K, 70, 238
Vivotoxin, 450

W

Water
absorption rate, 269-70
cell wall, 38
cyclic loss pattern, 268
deficit and physiology, 276-83
diurnal variation in plant, 268
factors affecting flux, 269-70
internal competition for, 268
loss control mechanisms, 271-76
loss in protoplasmic hydration, 277
loss rate, 269
moisture availability, 283
non-stomatal loss control, 273
pathway, 266-67
plant deficit effects, 265-87
seasonal deficits, 268
transport as catenary process, 271
use, 98
Waxes, 274
Weather and plant water level, 270
Wheat, geotropism in, 413
Wilt disease
external symptoms, 455-56
internal symptoms, 457-58
mechanism of, 461-62
nematodes and, 452
nitrogen role, 454
pectolytic enzymes and, 454
physiology of, 449-65
rhizosphere and, 452
root infection, 451-55
syndrome, 455-58
toxins, 449-51
vivotoxins, 449-51
Wilting, photosynthetic rate and, 280
Winkler method, 134
Woody plant growth, 382

X

Xanthophyll, 232
Xylan, 43
Xylem
hyperplasia, 457
ion movement, 107

Y

Yeast
amino acid in, 3, 5
sulfur metabolism in, 208
Yellowing, 456

Z

Zinc requirement, 71, 72, 81, 82

CUMULATIVE INDEXES

VOLUMES 3-12

INDEX OF CONTRIBUTING AUTHORS

A

Åberg, B., 8:153
Addicott, F. T., 6:211
Albaum, H., 3:35
Allen, P. J., 5:225
Arisz, W. H., 3:109
Arnon, D. I., 7:325
Ashton, F. M., 8:275
Axelrod, B., 7:267

B

Bandurski, R. S., 3:59
Barber, D. A., 11:127
Bawden, F. C., 3:171; 10:239
Bayley, S. T., 12:35
Beakbane, A. B., 8:217
Beevers, H., 7:267
Bennett, C. W., 7:143
Bentley, J. A., 9:47
Bergmann, W., 4:383
Bernstein, L., 9:25
Billings, W. D., 8:375
Blinks, L. R., 5:93
Bogorad, L., 9:417
Bollard, E. G., 11:141
Bonner, J., 3:59
Bonner, W. D., Jr., 8:427
Borthwick, H. A., 7:299
Boynton, D., 5:31
Bradbeer, C., 10:197
Braun, A. C., 5:133
Brauner, L., 5:163
Brian, P. W., 8:413
Briggs, G. E., 8:11
Brodie, H. W., 8:275
Brown, A. H., 4:23
Brown, J. C., 7:171
Broyer, T. C., 10:277
Bünning, E., 7:71
Burr, G. O., 8:275
Burris, R. H., 4:91; 10:301
Burström, H., 4:237

C

Cathey, H. M., 12:369
Chance, B., 9:449
Chandler, W. H., 10:1
Cheadle, V. I., 8:349
Chouard, P., 11:191
Christensen, J. J., 6:367
Clendenning, K. A., 8:137
Coleman, R. E., 8:275
Collander, R., 8:335
Conn, E. E., 3:307
Crafts, A. S., 4:253
Crane, F. L., 12:13
Currier, H. B., 8:349

D

Danielson, C. E., 7:215
De Vay, J. E., 6:367
Diehl, H. C., 3:149
Dimond, A. E., 6:329; 10:257
Doorenbos, J., 10:147
Duysens, L. N. M., 7:25

E

Eaton, F. M., 6:299
Embleton, T. W., 9:175
Emerson, R., 9:1
Epstein, E., 7:1
Esau, K., 8:349
Eyring, H., 5:271

F

Fawcett, C. H., 12:345
Fogg, G. E., 7:51
Folkes, B. F., 9:245
Frenkel, A. W., 4:23; 10:53
Fried, M., 12:91

G

Galston, A. W., 11:239
Gauch, H. G., 8:31
Gautheret, R. J., 6:433
Gibbs, M., 10:329
Goddard, D. R., 5:115
Goodwin, R. H., 4:283
Goodwin, T. W., 12:219
Gordon, S. A., 5:341
Greenidge, K. N. H., 8:237
Gruen, H. E., 10:405

H

Haagen-Smit, A. J., 4:305
Hackett, D. P., 10:113
Hagan, R. M., 12:265
Hansch, C., 6:157
Hartree, E. F., 4:115
Hartt, C. E., 8:275
Hayward, H. E., 9:25
Heinze, P. H., 5:205
Hendricks, S. B., 7:299
Hill, R., 4:115
Hoch, G., 12:155
Holly, K., 9:311
Horsfall, J. G., 10:257
Huber, B., 3:333

I

Iljin, W. S., 8:257

J

Jacobson, L., 3:189
Jaffe, L. F., 9:359
Jagendorf, A., 3:131
James, W. O., 4:59
Jones, W. W., 9:175
Joslyn, M. A., 3:149

K

Kamiya, N., 11:323
Kandler, O., 11:37
Ketchum, B. H., 5:55
Kok, B., 12:155
Kortschak, H. P., 8:275
Kramer, P. J., 6:253
Krasnovksy, A. A., 11:363
Krauss, R. W., 9:207
Kremers, R. E., 10:185
Kursanov, A. L., 7:401

L

Lang, A., 3:265
Laties, G. G., 10:87
Leeper, G. W., 3:1
Leopold, A. C., 9:281
Liverman, J. L., 6:177
Lumry, R., 5:271
Lundegårdh, H., 6:1
Lynch, R. S., 6:211

M

McCready, C. C., 9:311
McElroy, W. D., 5:1
Mapson, L. W., 9:119
Marrè, E., 12:195
Mason, H. L., 5:249
Melin, E., 4:325
Mercer, F., 11:1
Middleton, J. T., 12:431
Miller, C. O., 12:395
Mirov, N. T., 10:223
Mothes, K., 6:393
Muir, R. M., 6:157

N

Nason, A., 5:1
Neish, A. C., 11:55
Nicholas, D. J. D., 12:63
Nitsch, J. P., 4:199

O

Osterhout, W. J. V., 8:1
Overbeek, J. van, 3:87; 7:355
Overstreet, R., 3:189

P

Paech, K., 6:273
Pentzer, W. T., 5:205
Phinney, B. O., 11:411
Pirie, N. W., 3:171; 10:33
Pirson, A., 6:71
Pollard, J. K., 8:65
Purves, W. K., 11:239

Q

Quastel, J. H., 5:75

R

Rabinowitch, E., 3:299
Raney, F. C., 12:265
Ranson, S. L., 11:81
Raschke, K., 11:111
Ray, P. M., 9:81
Reinert, J., 10:441
Reuther, W., 9:175
Robertson, R. N., 8:11
Rogers, W. S., 8:217
Rosenberg, J. L., 8:115
Rufelt, H., 12:409
Russell, R. S., 11:127

S

Sadasivan, T. S., 12:449
Salisbury, F. B., 12:293
Samish, R. M., 5:183
Setterfield, G., 12:35
Shantz, E. M., 10:379
Shapiro, R. E., 12:91
Siegel, A., 11:277
Smith, L., 9:449
Spikes, J. D., 5:271
Stafford, H. A., 5:115
Stahmann, M. A., 6:351
Stanley, R. G., 10:223
Steemann Nielsen, E., 11:341
Steinberg, R. A., 9:151
Stern, H., 7:91
Steward, F. C., 8:65; 10:379
Stolwijk, J. A. J., 7:373
Stout, P. R., 5:249; 10:277
Stowe, B. B., 8:181
Stuart, N. W., 12:369
Stumpf, P. K., 3:17; 10:197

T

Takahashi, D., 8:275
Talling, J. F., 12:133
Tamiya, H., 8:309
Tanimoto, T., 8:275
Taylor, J. H., 12:327
Teas, H. J., 8:393
Teubner, F. G., 10:13
Thomas, M., 11:81
Thomas, M. D., 6:135
Toole, E. H., 7:299
Toole, V. K., 7:299
Torrey, J. G., 7:237
Tso, T. C., 9:151

U

Ulrich, A., 3:207
Ulrich, R., 9:385

V

Vaadia, Y., 12:265
Varner, J. E., 12:245
Veldstra, H., 4:151
Vennesland, B., 3:307
Virgin, H. I., 4:363
Virtanen, A. I., 12:1
Vishniac, W., 6:115

W

Walker, J. C., 6:351
Walter, H., 6:239
Wareing, P. F., 7:191
Wassink, E. C., 7:373
Webster, G. C., 6:43; 12:113
Wellensiek, S. J., 10:147
Went, F. W., 4:347
West, C. A., 11:411
Wildman, S. G., 3:131; 11:277
Williams, R. F., 6:25
Wittwer, S. H., 10:13
Wolken, J. J., 10:71
Wood, J. G., 4:1
Wood, R. K. S., 11:299
Woodford, E. K., 9:311

Y

Yamaki, T., 8:181
Yarwood, C. E., 7:115
Yemm, E. W., 9:245
Yocum, C. S., 11:25

Z

Zimmerman, M. H., 11:167

INDEX OF CHAPTER TITLES

VOLUMES 3-12

CELL STRUCTURE AND FUNCTION		
Physical Properties of Protoplasm	H. I. Virgin	4:363-82
Localization of Enzymes in the Cells of Higher Plants	D. R. Goddard, H. A. Stafford	5:115-32
The Structure of the Chloroplast	J. J. Wolken	10:71-86
The Submicroscopic Structure of the Cell	F. Mercer	11:1-24
Structure and Function of Mitochondria	F. L. Crane	12:13-34
Structure and Physiology of Cell Walls	G. Setterfield, S. T. Bayley	12:35-62
MINERAL NUTRITION		
Factors Affecting Availability of Inorganic Nutrients in Soils with Special Reference to Micronutrient Metals	G. W. Leeper	3:1-16
Physiological Bases for Assessing the Nutritional Requirements of Plants	A. Ulrich	3:207-28
Mechanism of Action of Micronutrient Elements in Enzyme Systems	W. D. McElroy, A. Nason	5:1-30
Nutrition by Foliar Application	D. Boynton	5:31-54
Mineral Nutrition of Phytoplankton	B. H. Ketchum	5:55-74
Soil Conditioners	J. H. Quastel	5:75-92
Functional Aspects in Mineral Nutrition of Green Plants	A. Pirson	6:71-114
Iron Chlorosis	J. C. Brown	7:171-90
Mineral Nutrition of Plants	H. G. Gauch	8:31-64
Mineral Nutrition of Tree Crops	W. Reuther, T. W. Embleton, W. W. Jones	9:175-206
Foliar Absorption of Mineral Nutrients	S. H. Wittwer, F. G. Teubner	10:13-32
The Macronutrient Elements	T. C. Broyer, P. R. Stout	10:277-300
Minor Mineral Nutrients	D. J. D. Nicholas	12:63-90
Soil-Plant Relationships in Ion Uptake	M. Fried, R. E. Shapiro	12:91-112
NITROGEN METABOLISM		
Leaf Proteins	S. G. Wildman, A. Jagendorf	3:131-48
Nitrogen Metabolism of Higher Plants	J. G. Wood	4:1-22
Nitrogen Metabolism	G. C. Webster	6:43-70
Nitrogen Fixation by Photosynthetic Organisms	G. E. Fogg	7:51-70
Plant Proteins	C. E. Danielson	7:215-36
Nitrogen Metabolism in Plants: Ten Years in Retrospect	F. C. Stewart, J. K. Pollard	8:65-114
The Metabolism of Amino Acids and Proteins in Plants	E. W. Yemm, B. F. Folkes	9:245-80
Leaf Proteins	N. W. Pirie	10:33-52
Nitrogen Nutrition	R. H. Burris	10:301-28
Nitrogen Fixation	C. S. Yocum	11:25-36
Some Aspects of the Structure and Behavior of Tobacco Mosaic Virus	A. Siegel, S. G. Wildman	11:277-98
Some Aspects of Amino Acid Synthesis in Plants and Related Subjects	A. I. Virtanen	12:1-12
Protein Synthesis	G. Webster	12:113-32
PHOTOSYNTHESIS		
Photosynthesis	E. Rabinowitch	3:229-64

Photosynthesis	A. H. Brown, A. W. Frenkel	4:23-58
The Photosynthetic Function of Pigments Other than Chlorophyll	L. R. Blinks	5:93-114
Photosynthesis	R. Lumry, J. D. Spikes, H. Eyring	5:271-340
Biochemical Aspects of Photosynthesis	W. Vishniac	6:115-34
Effect of Ecological Factors on Photosynthesis	M. D. Thomas	6:135-56
Energy Transformations in Photosynthesis	L. N. M. Duysens	7:25-50
Phosphorus Metabolism and Photosynthesis	D. I. Arnon	7:325-54
Photochemistry of Chlorophyll	J. L. Rosenberg	8:115-36
Biochemistry of Chloroplasts in Relation to the Hill Reaction	K. A. Clendenning	8:137-52
The Quantum Yield of Photosynthesis	R. Emerson	9:1-24
Cytochromes in Plants	L. Smith, B. Chance	9:449-82
Light Induced Reactions of Bacterial Chromatophores and Their Relation to Photosynthesis	A. W. Frenkel	10:53-70
The Structure of the Chloroplast	J. J. Wolken	10:71-86
Energy Transfer Through Phosphorylation Mechanisms in Photosynthesis	O. Kandler	11:37-54
Productivity of the Oceans	E. Steemann Nielsen	11:341-62
The Primary Processes of Photosynthesis in Plants	A. A. Krasnovsky	11:363-410
Photosynthesis Under Natural Conditions	J. F. Talling	12:133-54
Photosynthesis	G. Hoch, B. Kok	12:155-94
Biosynthesis and Function of Carotenoids	T. W. Goodwin	12:219-44
RESPIRATION		
Glycolytic Enzymes in Higher Plants	P. K. Stumpf	3:17-34
The Use of Respiratory Inhibitors	W. O. James	4:59-90
Mechanisms of Carbohydrate Breakdown in Plants	B. Axelrod, H. Beevers	7:267-98
Soluble Oxidases and Their Functions	W. D. Bonner, Jr.	8:427-52
Cytochromes in Plants	L. Smith, B. Chance	9:449-82
Respiratory Mechanisms in Higher Plants	D. P. Hackett	10:113-46
Metabolism of Carbon Compounds	M. Gibbs	10:329-78
Crassulacean Acid Metabolism	S. L. Ranson, M. Thomas	11:81-110
Structure and Function of Mitochondria	F. L. Crane	12:13-34
Phosphorylation in Higher Plants	E. Marre	12:195-218
GENERAL METABOLISM		
Metabolism of Phosphorylated Compounds in Plants	H. Albaum	3:35-58
Physiological Aspects of Low Temperature Preservation of Plant Products	M. A. Joslyn, H. C. Diehl	3:149-70
Carboxylating Enzymes in Plants	B. Vennesland, E. E. Conn	3:307-32
Organic Acids in Plant Metabolism	R. H. Burris	4:91-114
Hematin Compounds in Plants	R. Hill, E. F. Hartree	4:115-50
The Biogenesis of Terpenes	A. J. Haagen-Smit	4:305-24
The Plant Sterols	W. Bergmann	4:383-426
Mechanism of Action of Micronutrient Elements in Enzyme Systems	W. D. McElroy, A. Nason	5:1-30
Postharvest Physiology of Fruits and Vegetables	W. T. Pentzer, P. H. Heinze	5:205-24
Colour Development in Flowers	K. Paech	6:273-98
Physiology of Alkaloids	K. Mothes	6:393-432
Mechanisms of Carbohydrate Breakdown in Plants	B. Axelrod, H. Beevers	7:267-98
Soluble Oxidases and Their Functions	W. D. Bonner, Jr.	8:427-52

Metabolism of Ascorbic Acid in Plants: Part I. Function	L. W. Mapson	9:119-50
Postharvest Physiology of Fruits	R. Ulrich	9:385-416
The Biogenesis of Flavonoids	L. Bogorad	9:417-48
The Lignins	R. E. Kremers	10:185-96
Fat Metabolism in Higher Plants	P. K. Stumpf, C. Bradbeer	10:197-222
Metabolism of Carbon Compounds	M. Gibbs	10:329-78
Biosynthetic Pathways of Aromatic Compounds	A. C. Neish	11:55-80
Crassulacean Acid Metabolism	S. L. Ranson, M. Thomas	11:81-110
Pectic and Cellulolytic Enzymes in Plant Disease	R. K. S. Wood	11:299-322
Biosynthesis and Function of Carotenoids	T. W. Goodwin	12:219-44
Biochemistry of Senescence	J. E. Varner	12:245-64
Physiology of Mitosis and Meiosis	J. H. Taylor	12:327-44
WATER RELATIONS		
The Water Economy and the Hydrature of Plants	H. Walter	6:239-52
Water Relations of Plant Cells and Tissues	P. J. Kramer	6:253-72
Drought Resistance in Plants and Physiological Processes	W. S. Iljin	8:257-74
Heat Transfer Between the Plant and the Environment	K. Raschke	11:111-26
The Relationship Between Salt Uptake and the Absorption of Water by Intact Plants	R. S. Russell, D. A. Barber	11:127-40
Plant Water Deficits and Physiological Processes	Y. Vaadia, F. C. Raney, R. M. Hagan	12:265-92
Physiology of Wilt Disease	T. S. Sadasivan	12:449-68
ABSORPTION AND TRANSLOCATION		
Transport of Organic Compounds	W. H. Arisz	3:109-30
Mechanisms of Ion Absorption by Roots	R. Overstreet, L. Jacobson	3:189-206
Nutrition by Foliar Application	D. Boynton	5:31-54
Mechanisms of Absorption, Transport, Accumulation, and Secretion of Ions	H. Lundegårdh	6:1-24
Redistribution of Mineral Elements During Development	R. F. Williams	6:25-42
Mineral Nutrition of Plants: Mechanisms of Uptake and Transport	E. Epstein	7:1-24
Absorption and Translocation of Plant Regulators	J. van Overbeek	7:355-72
Apparent Free Space	G. E. Briggs, R. N. Robertson	8:11-30
Ascent of Sap	K. N. H. Greenidge	8:237-56
Permeability of Plant Cells	R. Collander	8:335-48
Physiology of Phloem	K. Esau, H. B. Currier, V. I. Cheadle	8:349-74
Foliar Absorption of Mineral Nutrients	S. H. Wittwer, H. G. Teubner	10:13-32
Active Transport of Salt into Plant Tissue	G. G. Laties	10:87-112
The Relationship Between Salt Uptake and the Absorption of Water by Intact Plants	R. S. Russell, D. A. Barber	11:127-40
Transport in the Xylem	E. G. Bollard	11:114-66
Transport in the Phloem	M. H. Zimmermann	11:167-90
Physics and Chemistry of Protoplasmic Streaming	N. Kamiya	11:323-40
GROWTH AND DEVELOPMENT		
Studies of the Physiology, Pharmacology, and Biochemistry of the Auxins	J. Bonner, R. S. Bandurski	3:59-86

Agricultural Application of Growth Regulators and Their Physiological Basis	J. van Overbeek	3:87-108
Physiology of Flowering	A. Lang	3:265-306
The Relation of Chemical Structure to Biological Activity in Growth Substances	H. Veldstra	4:151-98
The Physiology of Fruit Growth	J. P. Nitsch	4:199-236
Physiology of Root Growth	H. Burström	4:237-52
Herbicides	A. S. Crafts	4:253-82
The Effect of Temperature on Plant Growth	F. W. Went	4:347-62
The Physiology of Plant Tumors	A. C. Braun	5:133-62
Tropisms and Nastic Movements	L. Brauner	5:163-82
Dormancy in Woody Plants	R. M. Samish	5:183-204
Occurrence, Formation and Inactivation of Auxins	S. A. Gordon	5:341-78
Chemical Constitution as Related to Growth Regulator Action	R. M. Muir, C. Hansch	6:157-76
The Physiology of Flowering	J. L. Liverman	6:177-210
Physiology of Abscission	F. T. Addicott, R. S. Lynch	6:211-38
Tissue Culture	R. J. Gautheret	6:433-84
Endogenous Rhythms in Plants	E. Bünning	7:71-90
The Physiology of Cell Division	H. Stern	7:91-114
Photoperiodism in Woody Plants	P. F. Wareing	7:191-214
Physiology of Root Elongation	J. G. Torrey	7:237-66
Physiology of Seed Germination	E. H. Toole, S. B. Hendricks, H. A. Borthwick, V. K. Toole	7:299-324
Absorption and Translocation of Plant Regulators	J. van Overbeek	7:355-72
Effects of Light Quality on Plant Growth	E. C. Wassink, J. A. J. Stolwijk	7:373-400
Auxin Relations in Roots	B. Åberg	8:153-80
The History and Physiological Action of the Gibberellins	B. B. Stowe, T. Yamaki	8:181-216
Mass Culture of Algae	H. Tamiya	8:309-34
Effects of Antibiotics on Plants	P. W. Brian	8:413-26
The Naturally Occurring Auxins and Inhibitors	J. A. Bentley	9:47-80
Destruction of Auxin	P. M. Ray	9:81-118
Auxin Uses in the Control of Flowering and Fruiting	A. C. Leopold	9:281-310
Herbicides	E. K. Woodford, K. Holly, C. C. McCready	9:311-58
Morphogenesis in Lower Plants	L. F. Jaffe	9:359-84
Photoperiodic Control of Floral Induction	S. J. Wellensiek, J. Doorenbos	10:147-84
The Chemical Regulation of Growth (Some Substances and Extracts which Induce Growth and Morphogenesis)	F. C. Steward, E. M. Shantz	10:379-404
Auxins and Fungi	H. E. Gruen	10:405-40
Phototropism and Phototaxis	J. Reinert	10:441-58
Vernalization and Its Relation to Dormancy	P. Chouard	11:191-238
The Mechanism of Action of Auxin	A. W. Galston, W. K. Purves	11:239-76
Gibberellins as Native Plant Growth Regulators	B. O. Phinney, C. A. West	11:411-36
Structure and Physiology of Cell Walls	G. Setterfield, S. T. Bayley	12:35-62
Biochemistry of Senescence	J. E. Varner	12:245-64
Photoperiodism and the Flowering Process	F. B. Salisbury	12:293-326
Physiology of Mitosis and Meiosis	J. H. Taylor	12:327-44
Indole Auxins	C. H. Fawcett	12:345-68
Applied Aspects of the Gibberellins	N. W. Stuart, H. M. Cathey	12:369-94
Kinetin and Related Compounds in Plant Growth	C. O. Miller	12:395-408

Geotropism in Roots and Shoots	H. Rufelt	12:409-30
STRESS PHYSIOLOGY		
Physiological Aspects of Low Temperature Preservation of Plant Products	M. A. Joslyn, H. C. Diehl	3:149-70
Drought Resistance in Plants and Physiological Processes	W. S. Iljin	8:257-74
Physiology of Salt Tolerance	L. Bernstein, H. C. Hayward	9:25-46
Plant Water Deficits and Physiological Processes	Y. Vaadia, F. C. Raney, R. M. Hagan	12:265-92
Photochemical Air Pollution Damage to Plants	J. T. Middleton	12:431-48
PATHOLOGICAL PHYSIOLOGY		
Physiology of Virus Diseases	F. C. Bawden, N. W. Pirie	3:171-88
The Physiology of Plant Tumors	A. C. Braun	5:133-62
Physiological Aspects of Fungus Diseases of Plants	P. J. Allen	5:225-48
Pathogenesis in the Wilt Diseases	A. E. Dimond	6:329-50
Chemical Nature of Disease Resistance in Plants	J. C. Walker, M. A. Stahmann	6:351-66
Adaptation of Plant Pathogen to Host	J. J. Christensen, J. E. DeVay	6:367-92
Obligate Parasitism	C. E. Yarwood	7:115-42
Biological Relations of Plant Viruses	C. W. Bennett	7:143-70
Effects of Antibiotics on Plants	P. W. Brian	8:413-26
Physiology of Virus Diseases	F. C. Bawden	10:239-56
Plant Chemotherapy	A. E. Dimond, J. G. Horsfall	10:257-76
Pectic and Cellulolytic Enzymes in Plant Disease	R. K. S. Wood	11:299-322
Physiology of Wilt Disease	T. S. Sadasivan	12:449-68
SPECIAL TOPICS		
Tree Physiology	B. Huber	3:333-46
Fluorescent Substances in Plants	R. H. Goodwin	4:283-304
Physiology of Mycorrhizal Relations in Plants	E. Melin	4:325-46
Tropisms and Nastic Movements	L. Brauner	5:163-82
The Role of Plant Physiology in Plant Geography	H. L. Mason, P. R. Stout	5:249-70
Physiology of the Cotton Plant	F. M. Eaton	6:299-328
Recent Advances in Plant Physiology in the U. S. S. R.	A. L. Kursanov	7:401-36
The Use of Aquatic Plants in the Study of Some Fundamental Problems	W. J. V. Osterhout	8:1-10
Stock and Scion Relations	W. S. Rogers, A. B. Beakbane	8:217-36
The Sugarcane Plant	G. O. Burr, C. E. Hartt, H. W. Brodie, T. Tanimoto, H. P. Kortschak, D. Takahashi, F. M. Ashton, R. E. Coleman	8:275-308
Physiological Ecology	W. D. Billings	8:375-92
Physiological Genetics	H. J. Teas	8:393-412
Physiology of the Tobacco Plant	R. A. Steinberg, T. C. Tso	9:151-74
Physiology of the Fresh-Water Algae	R. W. Krauss	9:207-44
Plant Physiology and Horticulture	W. H. Chandler	10:1-12
The Pine Tree	N. T. Mirov, R. G. Stanley	10:223-38
Phototropism and Phototaxis	J. Reinert	10:441-58
Physics and Chemistry of Protoplasmic Streaming	N. Kamiya	11:323-40
Productivity of the Oceans	E. Steemann Nielsen	11:341-62